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# Bacterial Degradation of Nitrogenous Energetic Compounds (NEC) in Coastal Waters and Sediments

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Once released in the environment, either through detonation, casing breakage, or by slow leaks from unexploded ordnance (UXO), nitrogenous energetic compounds (NEC, such as TNT, HMX, RDX) may sorb onto particulates, partition to dissolved organic matter, or remain dissolved in aqueous media. Our hypothesis was that NEC would be transient in coastal ecosystems. This was based primarily on the understanding that microbial growth in these systems is typically nitrogen-limited and there are few examples of nitrogen based organic compounds that are not rapidly metabolized in these environments. During 14 sampling events in coastal waterways from 2002 to 2007, we measured TNT mineralization rates in surface sediment and water samples that were often the same as, or within one order of magnitude of, the rate of total heterotrophic bacterial metabolism. These rates were often similar to those of other organic compounds that are transient in natural ecosystems due to their use in bacterial metabolism, such as petroleum hydrocarbons and amino acids.

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not detected relative to sections where it was detected, PAH mineralization rates were higher among the latter sections.

**Table 14.** TNT mineralization was measured on sediment samples (68 total) from the Chesapeake Bay (35), San Francisco (10) and the Ala Wai canal, HI (11). Stations with detectable TNT mineralization rates were compared with those that were below the detection limit. Average (AVG) bacterial production and mineralization rates (μg C $kg^{-1}$ $d^{-1}$) for other organic compounds were generally higher for samples that had measurable TNT mineralization with the notable exception of DNT.

**Table 15.** TNT mineralization rate (μg C $kg^{-1}$ $d^{-1}$) as a function of bacterial production (μg C $kg^{-1}$ $d^{-1}$) was determined as a relative measure of the ability of a bacterial assemblage to metabolize TNT for core segments from San Francisco and Chesapeake Bays and Hawaii.

**Table 16.** Pacific Ocean seawater was sampled pre and post an underwater demolition detonation with 10 lbs. of TNT or RDX. There was little difference in bacterial production (μg C $L^{-1}$ $d^{-1}$) because of the detonation of TNT, but production roughly doubled in the post RDX detonation sample incubated in either ambient light or dark.

**Table 17.** Pacific Ocean seawater was sample pre and post an underwater demolition detonation with 10 lbs. of TNT. There was little difference in bacterial production, TNT mineralization or uptake rate (μg C $L^{-1}$ $d^{-1}$) as a result of the detonation (AVG: average; SD, one standard deviation). Incubation under ambient laboratory light increased both bacterial production and TNT uptake (μg C $L^{-1}$ $d^{-1}$); mineralization was not measured under dark conditions. Added 0.176 ppm of TNT.

# BACTERIAL DEGRADATION OF NITROGENOUS ENERGETIC COMPOUNDS IN COASTAL WATERS AND SEDIMENTS

**1.0 Introduction** The fate and transformation of 2,4,6-Trinitrotoluene (TNT) has been studied extensively in terrestrial and freshwater systems (Spain et al. 2000, Esteve-Nunez et al. 2001), however, similar investigations in the marine environment have been limited (Darrach et al. 1998, Lotofu et al. 2001). Marine systems are particularly important given historical and current ordnance disposal and training activities, which may liberate energetic compounds through detonation or slow release from unexploded ordnance (UXO). Though nitrogenous energetics persist in soil, freshwater sediments, and groundwater (Spain et al. 2000), preliminary investigations into their fate in marine systems have demonstrated a rapid biological and photochemical degradation. These conflicting results may directly relate to the ecosystem studied. Heterotrophic bacteria in terrestrial and groundwater environments are widely known to be phosphorus-limited, whereas the growth of marine microbiota is nitrogen limited (Vitousek and Howarth 1991, Carpenter and Capone 1983). Although TNT is not commonly found in nature, it can supply a valuable, growth-limiting nutrient to estuarine and marine ecosystems. This may be due to a denitration step in TNT transformation that liberates either $NO_3^-$ or $NH_4^+$ depending on oxygen availability (Spain et al. 2000). Additionally, genetic evidence supports TNT degradation by bacteria that metabolize other types of naturally occurring organic matter (polycyclic aromatic hydrocarbons (PAHs, lignin; Suen et al. 1996). We hypothesized that nitrogenous energetics will be transient in nitrogen-limited environments, such as coastal waters and sediments. In this report, we demonstrate rapid TNT biodegradation in marine systems. If proven to the extent that these findings are accepted by EPA, they have dramatic implications for lowering Navy UXO recovery and compliance costs and ecological damage liability. Currently, there are no acceptable methods for energetics cleanup in estuarine sediments, as even dredge and haul practices are complicated by the presence of UXO buried within the sediment. Land farming and composting are the most commonly reported strategies for soil cleanup, while for groundwater cleanup, slurry bioreactors (Steffan and Drew 1995), binding to organics (Fant et al. 2001), and reduction by Zero Valent Iron (ZVI) and related compounds have been proposed or attempted at the pilot scale (for reviews see Peres and Agathos 2000, Rodgers and Bunce 2001).

**1.1 Abiotic** Once released, either through detonation, casing breakage, or by slow leaks from UXO, TNT may sorb onto particulates, partition to dissolved organic matter, or remain dissolved in aqueous media. TNT has nitro groups ($NO_2^-$) on its ring structure that are polarizing and form hydrogen bonds with water. This characteristic increases the water solubility of TNT and its affinity for charged surfaces (Haderlein et al. 2000, van Beelen and Burris 1995, Lotufo et al. 2001). Adsorption of nitroaromatics (Haderlein and Schwarzenbach 1993, Haderlein et al. 1996, Weissmahr et al. 1997, Daun et al. 1998, Weissmahr et al. 1999) and nitramines (Leggett 1985, Sheremata et al. 2001, Brannon et al. 2003, Monteil-Rivera et al. 2003) to clays, specifically phyllosilicates, was determined to dominate sorptive processes in aqueous suspensions and groundwater. With smectite clays, TNT nitro group binding essentially replace the calcium in the clay matrix, thus integrating the energetic (Li et al. 2004). Under anoxic conditions in water, TNT nitro groups can be reduced to amines (Barrows et al. 1996) affecting its reactivity (Achtnich et al. 2000). The presence of reduced colloidal or particulate iron (ZVI) can also reduce these nitro groups and enhance both the reactivity and TNT mineralization in aqueous environments (Agrawal and Tratnyek 1996, Devlin et al. 1998,

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Brannon et al. 2000) though transformation rates based on laboratory experiments may overestimate the expected rates in the field (Bandstra et al. 2005). In addition to ZVI, zinc was also found to reduce TNT and longer-term reactivity was enhanced by the presence of corrosion promoters like chloride (Hernandez et al. 2004).

TNT has a low octanol-water partition coefficient ($K_{ow}$), indicating a limited affinity for surface-active organic matter relative to other higher $K_{ow}$ organic contaminants (*e.g.*, PAHs and PCBs (Karickhoff, 1984, Schwarzenbach et al, 1993)). However, significant differences in TNT sorption behavior were observed between low (0.02%) and high (8%) organic matter soils (Sheremata et al. 1999). Absorption onto or into natural organic matter (NOM) has been identified as a significant removal process for organic contaminants in environments where NOM is greater than >0.2% (Schwarzenbach et al. 1993), which might be expected in estuarine systems. Many terrestrial and groundwater studies identify the association of TNT and particulate organic matter (POM) as an irreversible binding (Selim et al. 1995, Comfort et al. 1995, Pennington et al. 1995, Achtnich et al., 1999, Pennington et al., 1999), but in the presence of live bacterial assemblages, it would be difficult to differentiate radiolabel incorporated into bacterial macromolecules from that covalently binding to POM. The presence of colloidal and dissolved organic matter (DOM) may also influence the fate of TNT in the marine water column and sedimentary porewater, as indicated in previous investigations on the enhanced water solubility of several organic pollutants by dissolved humic and fulvic acids (Chiou et al. 1986). Dissolved humic acids decreased the aqueous concentration of TNT and its metabolites in adsorption experiments under anaerobic conditions (Daun et al. 1998); however, an investigation using topsoil-derived DOM produced conflicting results, with no changes in the apparent nitroaromatic solubility (Sheremata et al. 1999). Using soil composting material, Thorn and coworkers (Thorn and Kennedy 2002, Thorn et al. 2002) found that TNT was reduced to nitramines and then covalently bound to humins in soil and this binding was increased by the presence of peroxidases. The majority of these previous investigations have focused predominantly on soil and groundwater environments that have limited available dissolved and particulate organic material (Haderlein et al. 1996, Weissmahr et al. 1999). Estuarine sediments have significant differences in organic geochemistries (Hedges and Oades 1997), which may limit the extrapolation of TNT behavior from terrestrial systems to marine systems.

**1.2 Biotic – heterotrophic bacteria** The rate of TNT attenuation below the photic zone in coastal waters and sediments may be largely controlled by heterotrophic bacterial metabolism. Virtually all of the published information on bacterial metabolism of nitrogenous energetic compounds (NEC) is derived from work in terrestrial soil (e.g. Drzyzga et al. 1999, Steffan and Drew 1995), groundwater (e.g. Krumholz et al. 1997) and freshwater systems (e.g. Spain et al. 2000). These studies provide a substantial amount of information on enzymatic pathways (see review by Peres and Aganthos 2000), production of intermediates or dead end products (Drzyzga et al. 1998), strain identification (Lessner et al. 2002, Smets and Mueller 2001) and some genetic information on the natural assemblage (Fuller and Manning 1998). Typically, some combination of aerobic and anaerobic metabolism is required for near complete transformation of TNT carbon and this often starts with denitration prior to ring cleavage (Fiorella and Spain 1997). There are also many reports of incomplete metabolism resulting in the production of intermediates or dead end by-products that may be reactive with natural organic matter (*e.g.* humics) (Knicker et al. 1999, Bruns-Nagel et al. 2000, Thorn et al. 2002).

These partial degradation products may be more toxic than the parent compound, which may result in increased risk to the ecosystem. There are also some early reports on the use of recombinant bacteria for TNT metabolism (Duque et al. 1993). To date, most work on NEC transformation by bacteria has been performed with environmental isolates rather than natural assemblages.

TNT biodegradation has been reported for a wide variety of bacterial isolates cultured from freshwater or terrestrial environments including *Pseudomonas aeruginosa* (Alvarez et al. 1995, Kalafut et al. 1998, Oh et al. 2003), *P. fluorescens* (Pak et al. 2000), *P. pseudoalcaligenes* (Fiorella, and Spain 1997), *Pseudomonas* sp. (Haïdour and Ramos 1996), anaerobic *Desulfovibrio* sp. and an aerobic *Serratia* sp. (Drzyga et al. 1998), *Bacillus sp., and Staphylococcus sp*. (Kalafut et al. 1998), actinomycetes (Pasti-Grigsby et al. 1996), *Enterobacter cloacae* (French et al. 1998), *Klebsiella* sp. (Kim et al. 2002), *Clostridium thermoaceticum* (Huang et al 2000), and *C. acetobutylicum* (Watrous et al. 2003). There are fewer reports of 2,4-dinitrotoluene biodegradation by cultured *Arthrobacter* sp. (Tope et al. 1999) *Alcaligenes* sp. (Smets and Mueller 2001), and *Burkholderia cepacia* (Johnson, et al. 2002), and 2-amino-4,6-dinitrotoluene by *P. aeruginosa* (Alvarez et al. 1995).

A common finding is that the $-NO_2$ groups of the TNT are reduced to $-NH_2$ or denitrated (*Bacillus* sp.; Kalafut et al. 1998) with extracellular release of the various aminotoluene partial degradation products. This represents dissimilatory transformation of TNT by bacteria where the energetic is either used as a terminal electron acceptor or is cometabolized by enzymes induced for other cellular functions. TNT metabolism in monoculture can be limited by nitroreductase inactivation by these partial degradation products (Riefler and Smets 2002) or by more generally described cytotoxicity (Fuller and Manning 1997). Reduction and cleavage of the aromatic ring of TNT has not been characteristic of most bacterial isolates studied to date (Zaripov et al. 2004). Enzymes involved in TNT metabolism include carbon monoxide dehydrogenase (*C. thermoaceticum*, Huang et al. 2000), NAD(P)H-dependent nitroreductase I (*Klebsiella* sp., Kim and Song 2005), NAD(P)H-dependent flavoprotein oxidoreductase (*P. fluorescens*, Pak et al. 2000), Fe-only hydrogenase (*C. acetobutylicum*; Watrous et al. 2003) and the flavin-dependent oxidoreductases related to the Old Yellow Enzyme family of yeast (Williams et al. 2004). These latter oxidoreductases did yield metabolic products that are indicative of reduction of the aromatic ring (Williams et al. 2004). Atypically, *Rhodococcus erythropolis* and a 4-nitrotoluene-utilizing *Mycobacterium* sp. possess reductive enzyme systems that catalyze ring hydrogenation without evidence of reductive TNT denitration (Vorbeck et al. 1998).

Heterotrophic bacteria have been reported to use TNT as a sole nitrogen source (*E. cloacae*; French et al. 1998), sole carbon source via nitrobenzene (*P. pseudoalcaligenes*; Fiorella and Spain 1997), with both the carbon and nitrogen incorporated into macromolecules (*Pseudomonas* sp.; Esteve-Nunez et al. 2000). Use as a sole carbon or nitrogen source represents assimilatory (or anabolic) use of energetics by bacteria resulting in the production of new biomass. Carbon incorporation can be relatively high (42%; Drzyzga et al. 1998) though the amount mineralized is typically very low (6% or less for *Klebsiella* sp.; Kim et al. 2002). Toluene can be produced from the denitration of TNT (Boopathy and Kulpa 1992). It can also be produced through the relatively simple genetic manipulation of transferring the *P. putida* TOL plasmid pWWO-Km to *Pseudomonas* sp. allowing the transconjugant to grow on TNT as a sole

source of carbon and nitrogen (Duque et al. 1993). Horizontal gene transfer efficiency of the TOL plasmid is very high, especially in biofilms, and particularly among non-culturable strains (Sorensen et al. 2005). Such plasmids are often unstable and lost upon exposure to the high nutrient culture conditions typically involved in isolating strains from the natural environment (Sorensen et al. 2005).

2,4-DNT can also be used as a sole carbon, nitrogen and energy source (*B. cepacia*; Johnson et al. 2002). The 2,4-DNT dioxygenase of *B. cepacia*, and the naphthalene dioxygenase enzyme system of *Pseudomonas* share a common ancestor (Suen et al. 1996) and appear to have evolved relatively recently based on the presence of extraneous transposable elements within the gene fragment (Johnson et al. 2002). Leungsakul et al. (2005) were able to use enzyme engineering strategies to enhance degradation of NECs including 2,5-DNT by *Burkholderia* sp. Relative to the amount of information on TNT biodegradation by cultured isolates, there are only scattered reports using mixed or natural assemblages and these include work on sludge (Kroger et al. 2004), groundwater and aquifer sediment (Krumholz et al. 1997), lake surface water (Spanggord et al. 1980, Talmage et al. 1999, Zeng et al. 2004), lake sediment (Boopathy and Kulpa 1994), and soils (Fuller et al. 1998, Miyares and Jenkins 2000, Siciliano et al. 1999). It seems difficult to imagine that natural assemblages of bacteria and protozoan grazers would be unable to mineralize NEC carbon and nitrogen given the importance of nitrogen as a growth-limiting nutrient in marine systems and the ubiquity of enzyme systems for metabolizing aromatic carbon compounds in nature:

> *"Although most organisms have detoxification abilities (i.e. mineralization, transformation and/or immobilization of pollutants), microorganisms, particularly bacteria, play a crucial role in biogeochemical cycles and in sustainable development of the biosphere. Next to glucosyl residues, the benzene ring is the most widely distributed unit of chemical structure in nature, and many of the aromatic compounds are major environmental pollutants. Bacteria have developed strategies for obtaining energy from virtually every compound under oxic or anoxic conditions (using alternative final electron acceptors such as nitrate, sulfate, and ferric ions). Clusters of genes coding for the catabolism of aromatic compounds are usually found in mobile genetic elements, such as transposons and plasmids, which facilitate their horizontal gene transfer and, therefore, the rapid adaptation of microorganisms to new pollutants. A successful strategy for in situ bioremediation has been the combination, in a single bacterial strain or in a syntrophic bacterial consortium, of different degrading abilities with genetic traits that provide selective advantages in a given environment."* (Diaz 2004)

Reports of TNT half lives in natural environmental samples range from 1.1 days (Miyares and Jenkins 2000) to weeks (Boopathy et al. 1997) to months (Talmage et al. 1999). Popesku et al. (2004) reported high removal rates but only 4% mineralization of TNT carbon by an oil-degrading assemblage during a long-term incubation (163 days). Degradation rates were temperature dependant in soil communities (Miyares and Jenkins 2000) and dependant on the availability of other forms of nitrogen and electron acceptors (Boopathy et al. 1997, Krumholz et al. 1997). Spain and coworkers reported use of 2,4-DNT and 2,6-DNT by soil assemblages (slurry reactors) as sole sources of carbon, nitrogen, and energy (Lendenmann et al. 1998, Nishino et al. 2000, Nishino et al. 1999, Zhang et al. 2000). There is little published information

on TNT degradation in seawater or marine sediments aside from the work of Carr and Nipper (2003) which found rapid TNT and 2,4-DNT transformation rates using Puget Sound water and sediment and an increase in heterotrophic activity by the natural assemblage which was attributed to TNT additions.

Exposuring soil assemblages to TNT can lead to a shift in the bacterial community structure (Fuller et al. 1998, Siciliano et al. 1999) and metabolic rate lowering (Zeng et al. 2004). There was a positive correlation between NEC concentrations and the presence of the genes responsible for denitrification (*nir*K, *nir*S or *nos*Z) in DNA extracted from the whole community, as well as in culturable strains isolated from the community, though denitrification rates decreased with increasing TNT concentrations (Siciliano et al. 2000). Using freshwater microbial assemblages, Zeng et al. (2004) found short-term exposure to TNT (90 min) had cytotoxic effects (lower plate counts and glucose mineralization rates) which could be reversed by exposure to light. The researchers concluded that the photodegradation products (e.g. 2-amino-4,6-DNT, 4-amino-2,6-DNT, and 3,5-dinitroaniline) were being used as growth substrates by the bacterial assemblage.

In addition to NEC catabolism by heterotrophic bacteria, fungi may play a role in degradation amongst the natural assemblage. Lignin is degraded by fungi using several key enzyme systems: manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase, which may degrade many different types of chemical bonds (Reddy 1995). These enzymes have also been shown to degrade PAHs, such as anthracene and phenanthrene (Pickard et al. 1999). Because aromatic compounds are synthesized by virtually all organisms, it is reasonable that anthropogenic compounds resembling natural aromatic compounds (amino acids, phenols, quinones; Fritsche and Hofrichter 2000) may be metabolized in microbial communities occurring where these compounds accumulate. Studies on fungal degradation of $^{14}C$ ring labeled TNT showed release of $^{14}CO_2$, evidence of aromatic ring breakage (Fernando et al. 1990). This aromatic ring is common to lignin, PAHs, and TNT and ability to cleave this subunit may be the reason that certain microbial communities are able to metabolize all three types of compounds.

In an effort to displace $^{14}C$-TNT covalently bound to soil compost, Gunnison et al. (1998) added ammonium to increase the bioavailability of TNT to natural microbial assemblages. Only 0.6% of the total $^{14}C$-TNT became mineralized in 30 d, however, 23% and 15% were mineralized from the lignocellulose and fulvic fractions. Their interpretation was that the ammonium caused the bound TNT to disassociate from the OM fractions, however, the nitrogen amendment could have also selected for heterotrophic bacteria or fungi that degrade all aromatic organic carbon without the need to disassociate the two components. Fungal-mediated TNT mineralization occurs only when fungi are lignolytic; i.e., when fungi produce lignin peroxidase (LiP) and manganese peroxidase (MnP), the lignin-degrading peroxidase enzymes (Hawari et al., 1999). Under these conditions, substantial carbon mineralization can occur (35%, Fernando et al. 1990, 30% Kim and Song 2003). Partial TNT degradation products like 4-hydroxylamino-2,6-DNT are potent competitive inhibitors of lignin peroxidase H8 of *P. chrysosporium* (Bumpus and Tatarko 1994, Michels and Gottschalk 1994), further evidence of the relationship between microbial degradation of NEC and lignin.

## 2.0 Material and methods

**2.1 Sampling** Subtidal stations were sampled from the Chesapeake and Delaware Bays (Figure 1), San Francisco Bay (Figure 2) the Ala Wai Canal, Oahu (Figure 3), Pearl Harbor and offshore of Oahu (Figure 4), Kahana Bay, Oahu (Figure 5), Delaware Bay (Figure 6) and the Gulf of Mexico (Figure 7). Surface sediments were collected using Smith MacIntyre (*R/V Point Sur*, *R/V Barnes*), Petite Ponar, Shipek (*R/V Cape Henlopen*), or Wilco benthic grabs. Gravity corers (Wilco) were used to sample the top 20 cm of sediment at stations in the lower Chesapeake Bay, San Francisco Bay, Pearl Harbor and the Ala Wai Canal. Divers were used to collect surface sediment offshore of Oahu. Surface (1 m below air-sea interface) and bottom water (1 m above sediment) were collected using a Sea Bird CTD with rosette or Go-Flo bottle. Chesapeake and Delaware Bay were sampled using the *R/V Cape Henlopen* and from shore, while the *R/V Point Sur* was used for the San Francisco Bay. Small watercraft were used for the Hawaii samplings. The *R/V Barnes* was used for sampling in the Gulf of Mexico. All data presented in this report were collected during 14 sampling events from 2002-2007 (Table 1). Gravity cores were sectioned every 2-3 cm for subsampling for mineralization or bacterial production in the depth profiles. Surface sediments and core slices were subsampled with a cut off 5 cm plastic syringe for the surveys of mineralization and a cut of 1 cm plastic syringe for bacterial production.

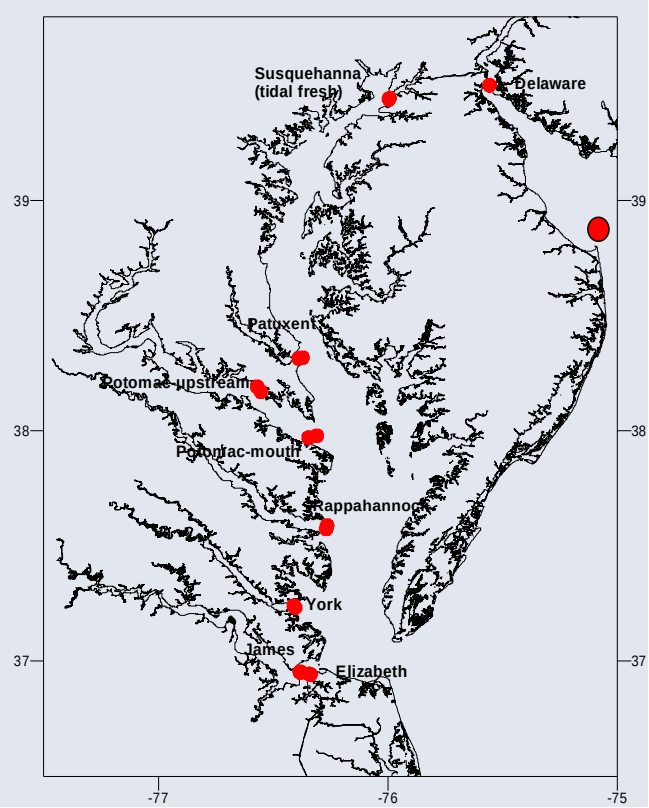


**Figure 1.** Chesapeake Bay station locations (●) for cruises in September 2002 and March of both 2004 and 2005.

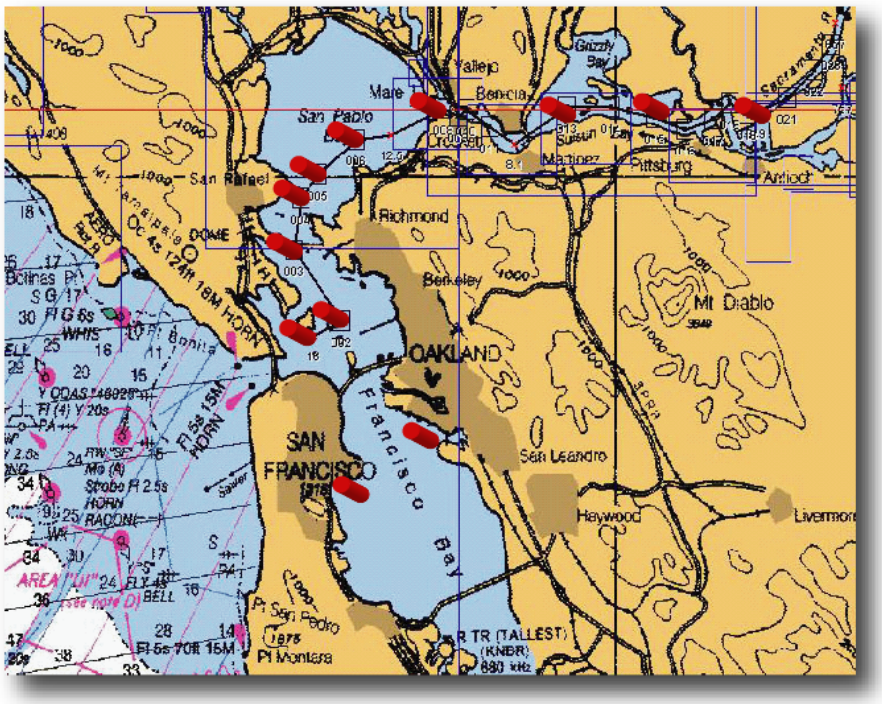


**Figure 2.** Sampling locations (●) for stations in the San Francisco Bay for May 2003.

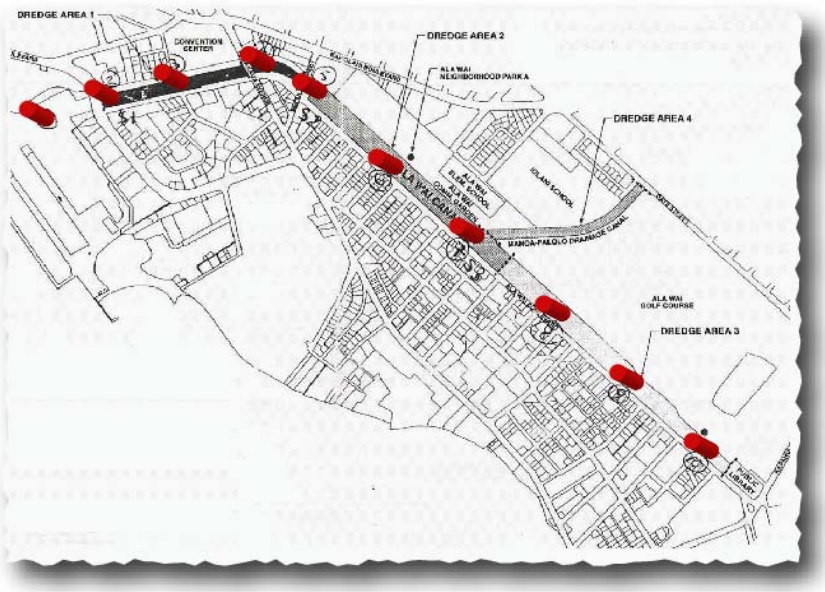


**Figure 3.** Eleven stations (●) were sampled in the Ala Wai Canal during July 2003.

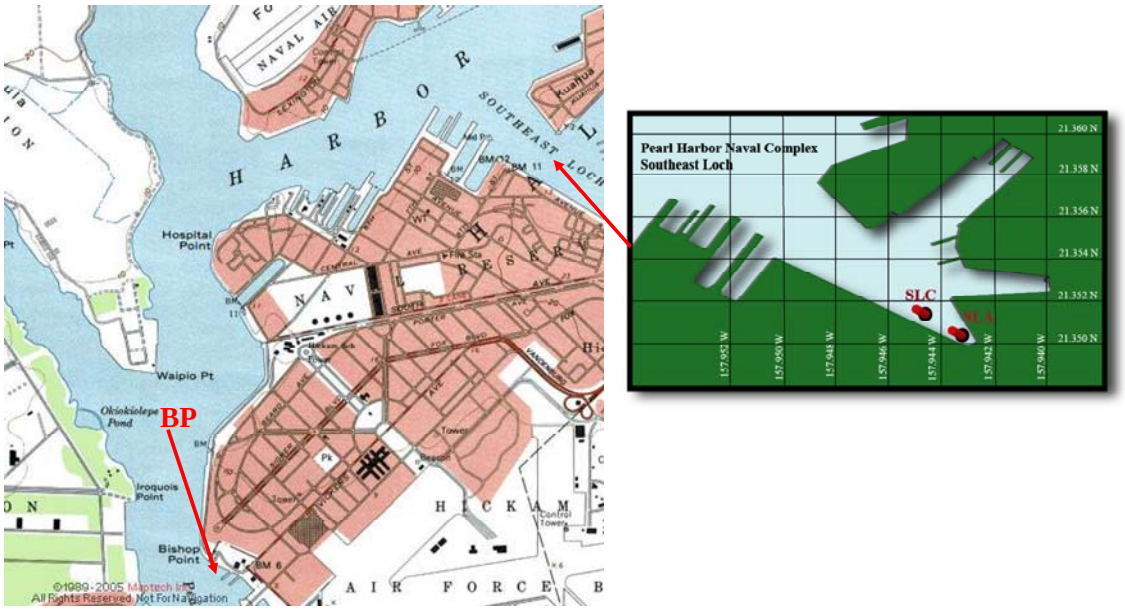


**Figure 4.** Depth profile cores were taken in Bishop's Point (BP) and Southeast Loch (SL) in Pearl Harbor, HI in December 2002.

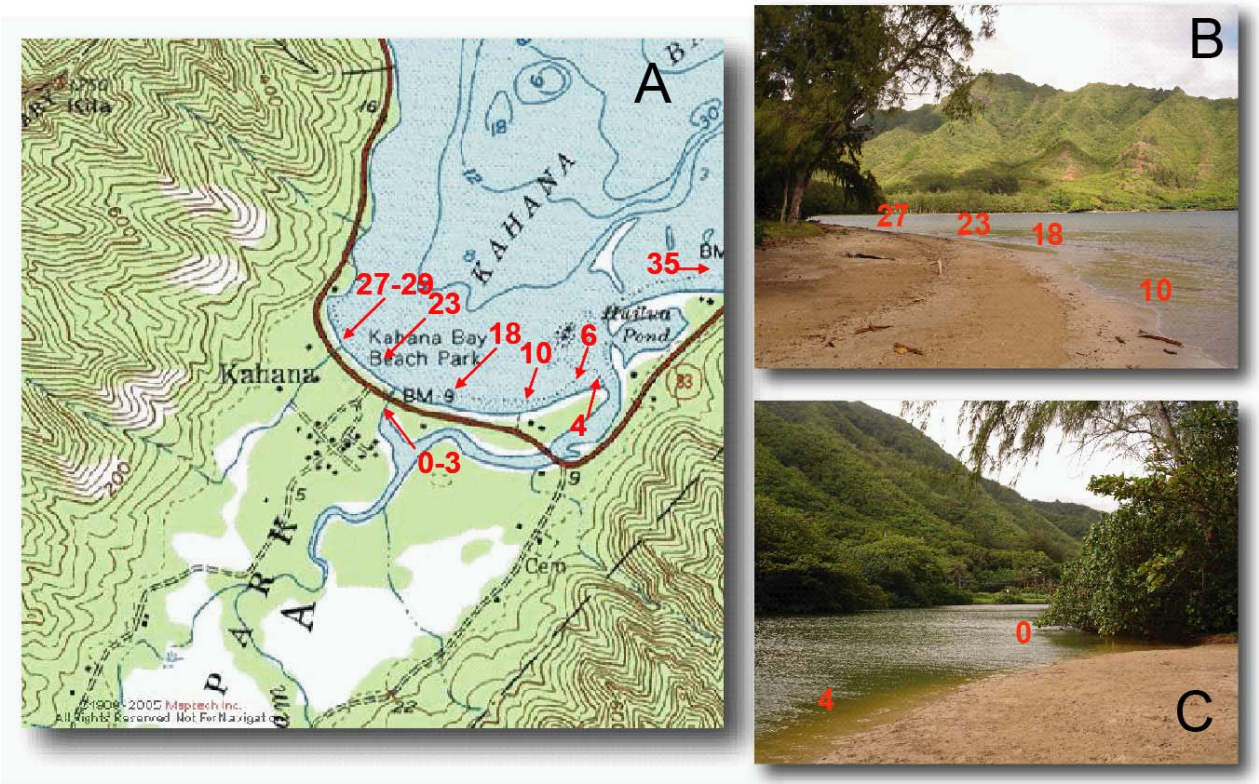


**Figure 5.** A) Sampling stations from salinity transect in Practical Salinity Units (PSU) in Kahana Bay on the windward side of Oahu in May and August 2006, and August 2007; B) at the 6 PSU station looking towards the 27 PSU station; and, C) looking towards the 0 PSU station.


**Figure 6.** Samples stations from near the mouth of the Delaware Bay in June 2006, F (0 psu), EST (18 PSU, *pictured top*), M (35 PSU).

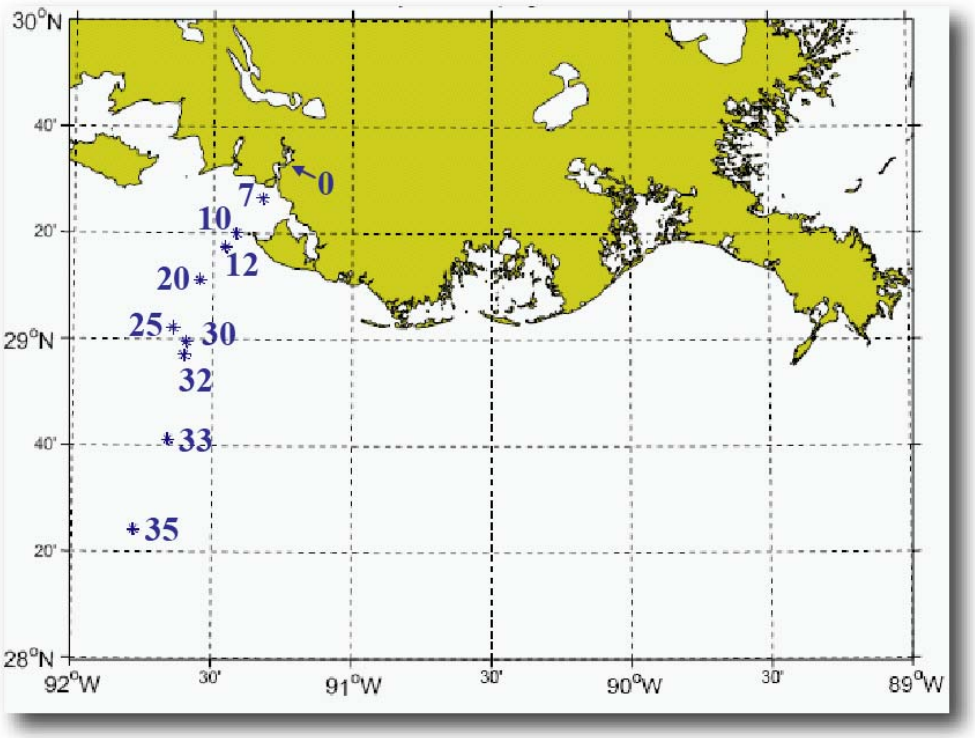


**Figure 7.** Sampling stations (*) from a salinity transect (PSU) in Gulf of Mexico (May 2007).

**Table 1.** Fourteen sampling cruises were performed in five ecosystems from 2002 to 2007.

| Ecosystem | Cruise | Stations |
|---|---|---|
| Chesapeake Bay | September 2002 | 17 |
| | March 2004 | 18 |
| | March 2005 | 12 |
| San Francisco Bay | May 2003 | 12 |
| Hawaii | December 2002 | 4 |
| | July 2003 | 11 |
| | May 2005 | 2 |
| | May 2006 | 5 |
| | August 2006 | 5 |
| | August 2007 | 5 |
| Delaware Bay | March 2005 | 1 |
| | June 2006 | 3 |
| | December 2006 | 3 |
| Gulf of Mexico | May 2007 | 19 |

**2.2 Mineralization** Organic carbon mineralization assays were initiated within three hours of sediment sample collection using a modification of Boyd et al. (1996) and Pohlman et al. (2002). As radiotracers, we used three sentinel PAHs: UL-$^{14}$C-naphthalene (spec. act: 18.6 mCi mmol$^{-1}$), 3-$^{14}$C-fluoranthene (45 mCi mmol$^{-1}$), and 9-$^{14}$C-phenanthrene (47 mCi mmol$^{-1}$; Sigma Chemical), as well as UL-$^{14}$C-TNT (spec. act.: 4 mCi mmol$^{-1}$), UL-$^{14}$C-2,4-DNT (55 mCi mmol$^{-1}$), UL-$^{14}$C-2,4-diaminotoluene (DAT; 55 mCi mmol$^{-1}$, American Radiochemicals Inc.), UL-$^{14}$C-catechol (1.8 mCi mmol$^{-1}$, Sigma Chemical), and UL-$^{14}$C-toluene (60 mCi mmol$^{-1}$, Sigma Chemical). They were added in separate incubations to surface sediment samples (1 mL wet volume) or 5 mL of seawater in 100×16 mm test tubes to a final concentration of about 0.2 μg g$^{-1}$ (depending on specific activity). For sediment samples, 0.5 mL of bottom water from the same station was filtered (0.22 μm nom. pore dia., Nuclepore polycarbonate) and added to make slurries. Samples were typically incubated for 24 h at *in situ* temperature in the dark and evolved $^{14}CO_2$ was captured on NaOH-soaked filter papers. $H_2SO_4$ was added to end incubations and to partition any remaining $CO_2$ into headspace of the tube and to the filter paper trap. The filter paper traps containing metabolized $^{14}CO_2$ were removed, radioassayed and subsequently used to calculate substrate mineralization. If detectable, ambient concentration of the specific compound was used to determine an isotope dilution factor.

**2.3 Heterotrophic bacterial production** The leucine incorporation method (Kirchman et al. 1985, Kirchman 1993, Smith and Azam 1992) was used to measure bacterial production as adapted by Montgomery et al. (1999). A 0.50 μL aliquot of wet surface sediment from each station was added to 2 mL microcentrifuge tubes with O-ring (Fisher; three experimental and one control) which were pre-charged with [$^3$H-4,5]-L-leucine (Amersham; 154 mCi mmol$^{-1}$). The sediment was extracted from the benthic grab sample or core section and added to the 2 mL tube using a 1 mL plastic syringe with the end cut off. One mL of 0.45 μm nom. pore dia. (Acrodisk, Gelman) filtered bottom water (collected <1 m above bottom) was then added to each tube to form a sediment slurry. Samples were incubated for 1 h at *in situ* temperatures and subsequently processed using TCA precipitation by the method of Smith and Azam (1992). A constant isotope dilution factor of 1000 was used for all samples. This was estimated from actual measurements of sediment dissolved free amino acids (Burdige and Martens 1990) and saturation experiment estimates (Tuominen 1995). One mL-syringed samples of wet sediment were dried at 50 $^o$C and used to covert production values to dry weight. Leucine incorporation rate was converted to bacterial carbon using factors determined by Simon and Azam (1989).

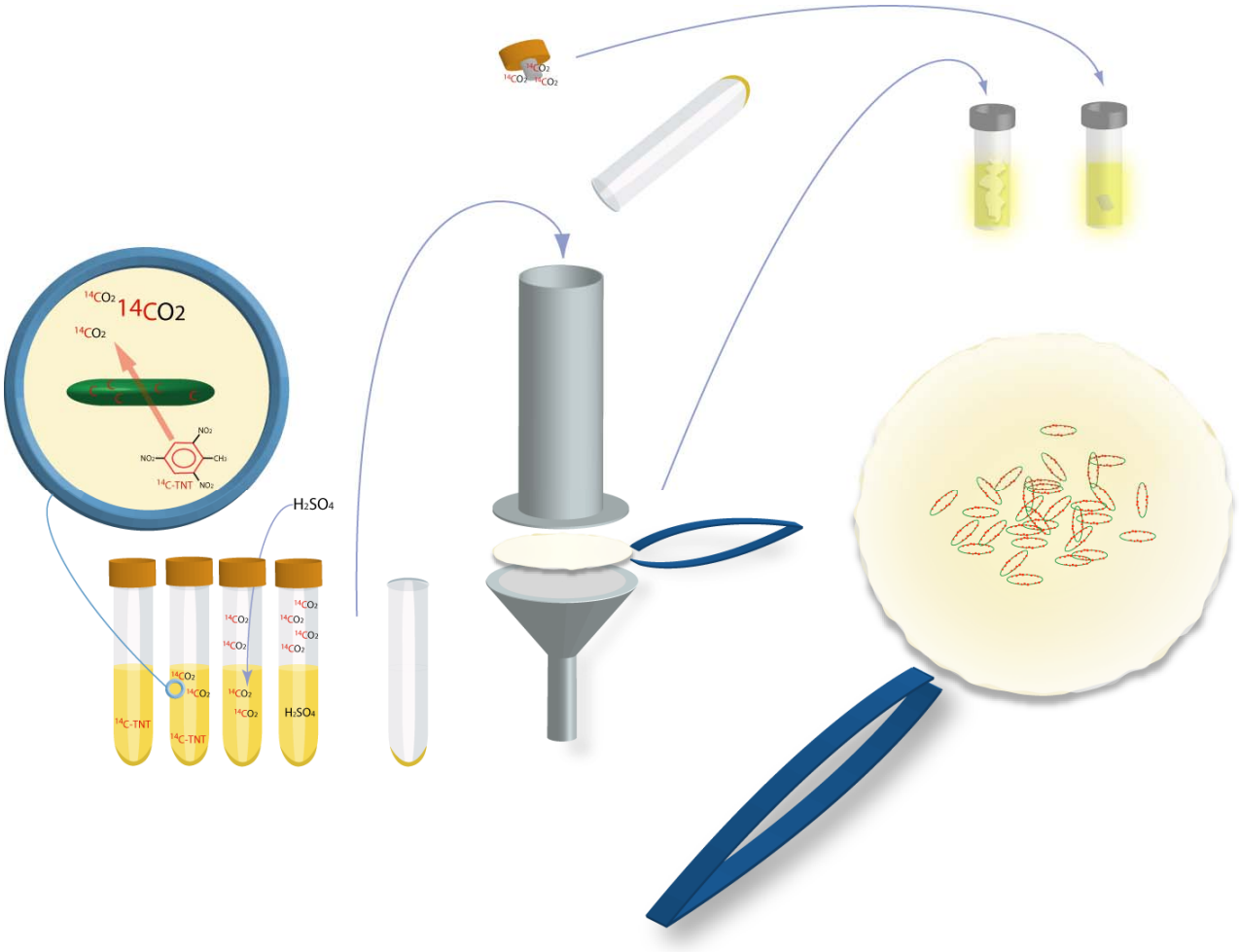


**Figure 8.** $^{14}$C-Radiolabeled energetics (TNT shown here) are incubated with the aqueous bacterial assemblage which incorporate some of the carbon into bacterial macromolecules. These are precipitated onto polycarbonate filter and then radioassayed.

**2.4 Incorporation into bacterial macromolecules** UL-$^{14}$C-TNT (spec. act.: 4 mCi mmol$^{-1}$), UL-$^{14}$C-2,4-DNT (55 mCi mmol$^{-1}$), UL-$^{14}$C-2,4-DAT (55 mCi mmol$^{-1}$, American Radiochemicals Inc.), were added in separate incubations to 5 mL of surface or bottom water in 100×16 mm test tubes to a final concentration of about 0.2 μg g$^{-1}$ (depending on specific activity). Samples were typically incubated for 48 h at *in situ* temperature in the dark prior to the addition of ice-cold TCA (final concn = 5%) and incubated an additional 30 min. Samples were then filtered (0.22 μm nom. pore dia., Nuclepore polycarbonate) prior to radioassay to determine the amount of energetic carbon had become incorporated into the bacterial macromolecule precipitate on the filter (Figure 8). Values for killed incubations (TCA added immediately to final concn of 5%) were subtracted from the values for the live incubations. Initially several different types of killing strategies were used (formalin, prefiltering, TCA) and there appeared to be little difference among the killed treatments.

**2.5 Contaminant concentration** Ambient PAH concentrations of 18 semi-volatile priority pollutants were determined. First, 10-15 g of sediment was dried with

diatomaceous earth and then extracted in methanol using accelerated solvent extraction. The extracts were concentrated under a $N_2$ stream (Speedvap) and analyzed by GC/MS (Fisher et al. 1997). *p*-Terphenyl-$d_{14}$ and 2-fluorobiphenyl were used as surrogate standards, following the method described in Pohlman et al. (2002). Energetic compounds were measured using standard EPA method 8330 and liquid chromatography mass spectrometry (LCMS).

## 3.0 Results and discussion

**3.1 Determine metabolic efficiency of NEC degradation** During the estuarine sediment surveys, we found that TNT mineralization was frequently more rapid than 2,4-DNT or 2,6-DAT mineralization at the same station and collection time. This could be due to a greater percentage of the bacterial community metabolizing TNT, more inefficient use of TNT by bacteria (lower % incorporation), or different cellular transport mechanism for TNT verses 2,4-DNT resulting in greater uptake and incorporation of TNT. First, the incorporation and mineralization rates of several nitrotoluenes were examined using $^{14}$C-TNT, $^{14}$C-DNT and $^{14}$C-DAT in Delaware Bay surface water. Then, $^{14}$C-TNT incorporation was measured over several salinities over a relatively small area of the lower Delaware Bay. These measurements were compared to heterotrophic bacterial production over a salinity gradient in the Kahana Bay estuary that was sampled three times over 16 months and included measurements for $^{14}$C-RDX and $^{14}$C-HMX. Finally, a larger survey was undertaken using $^{14}$C-TNT, $^{14}$C-RDX and $^{14}$C-HMX in the Gulf of Mexico.

Estuarine surface water (30 PSU) was collected from the mouth of the Delaware Bay in March 2005 and incubated with $^{14}$C-TNT, $^{14}$C-DNT and $^{14}$C-DAT to measure free-living bacterial incorporation and mineralization rates and growth efficiencies. Bacteria among the natural assemblage incorporated TNT and 2,6-DAT into macromolecules (TCA precipitate) at a similar rate over the course of the 24 h incubation (28.8 +/- 12.2 verses 24.5 +/- 2.2 $\mu g\ C\ L^{-1}\ d^{-1}$, respectively) but had a lower growth efficiency on TNT relative to 2,6-DAT and the mineralization rate was approximately six fold less for 2,6-DAT than TNT (3.1 +/- 0.9 verses 0.5 +/- 0.2 $\mu g\ C\ L^{-1}\ d^{-1}$; Table 2). That is, heterotrophic bacteria processed 2,6-DAT and TNT at about the same rate, but more of the 2,6-DAT carbon was incorporated into macromolecules and more of the TNT carbon was respired as $CO_2$.

Though there were differences in incorporation efficiencies among these compounds, they are still surprisingly high for organic carbon sources metabolized by a natural assemblage. One hypothesis generated from these results is that these compounds are metabolized efficiently because the N-C subunits of NEC are easily transformed into macromolecules like proteins and nucleic acids. Assimilatory nitrogen metabolism by bacteria may be the preferred transformation process rather than organic carbon oxidation of the aromatic ring for energy. Although the mineralization rates ($\mu g\ C\ L^{-1}\ d^{-1}$) were high relative to other organic carbon substrates, the percentage of assimilated TNT carbon that was mineralized was so low (ca. 10%) that it may actually be due to remineralization of bacterially incorporated $^{14}$C by protozoan grazers.

A salinity gradient from the Delaware Bay (0, 16, 34 PSU) was sampled during June 2006 and TNT mineralization rate was highest (1.11 +/- 0.37 $\mu g\ C\ L^{-1}\ d^{-1}$) at the highest salinity (34 PSU; Table 3). The relationship between salinity and TNT metabolism was further investigated at a site on the windward side of Oahu that had relatively little urban input

(surrounded by National Park lands) and had a distinct salinity gradient from freshwater to full strength seawater that could be sampled from shore. After a high rain event which produced extensive flooding in May 2006 at Kahana Bay, TNT incorporation rate was measured on the surface water natural assemblage and found to decrease with increasing salinity from 54.8 μg C $L^{-1}$ $d^{-1}$ at 0 PSU to 3.02 μg C $L^{-1}$ $d^{-1}$ at 35 PSU (Table 4). When the TNT incorporation rates were normalized for differences in heterotrophic bacterial metabolism among stations, there was an exponential decrease in the assemblage's ability to incorporate TNT carbon into bacterial macromolecules (Figure 9B). Note that the TNT incorporation rate was not directly correlated with changes in production along the salinity gradient (e.g. production increased at 18 and 28 PSU as incorporation decreased; Figure 9A). This argues against the idea that the incorporation rate measurements are the result of bacterial metabolic activity generating a TNT transformation product which then abiotically binds to humic material (and showing up in the bacterial macromolecule extract) as they would be expected to be a function of bacterial growth rate.

**Table 2.** Average (AVG) rates of TNT incorporation, mineralization (μg C $kg^{-1}$ $d^{-1}$) and growth efficiency (Incorporation + Mineralization/Incorporation*100) using Delaware Bay surface water (30 PSU) collected March 2005.

| Energetic | Rate (μg C $L^{-1}$ $d^{-1}$) | | % Growth Efficiency |
|---|---|---|---|
| | Incorporation | Mineralization | |
| TNT | 28.8 (12.2) | 3.1 (0.9) | 90.3 |
| DAT | 24.5 (2.2) | 0.5 (0.2) | 98.0 |
| DNT | 3.2 (0.2) | 1.1 (0.7) | 74.4 |

**Table 3.** Mineralization rates for three surface water samples from the Delaware Bay in June 2006.

| Salinity (psu) | Mineralization Rate (μg C $L^{-1}$ $d^{-1}$, AVG (SD)) |
|---|---|
| 0 (F) | 0.81 (0.37) |
| 16 (EST) | 0.53 (0.52) |
| 34 (M) | 1.11 (0.37) |

During August 2006 (no recent rain event), the same stations were sampled with very similar rates of TNT incorporation measured for the two lowest salinity stations (3 and 6 PSU)

and the midestuarine station (23 PSU). However, the marine station (35 PSU) had much higher TNT incorporation rate in the August sampling (46.3 μg C $L^{-1}$ $d^{-1}$) relative to the May sampling (3.02 μg C $L^{-1}$ $d^{-1}$). The 10 PSU station had the highest TNT incorporation rate of all Kahana Bay stations sampled in either May or August (121 μg C $L^{-1}$ $d^{-1}$; Table 5) and appeared to be in an area of convergence between water masses. While there appears to be some relationship between salinity and TNT metabolism, other factors, such as mixing and the presence of other refractory compounds (e.g. lignin, humics) present in areas of convergence, may also influence the TNT metabolizing bacterial assemblage.

**Table 4.** Average (AVG) rates of TNT incorporation, mineralization (μg C $L^{-1}$ $d^{-1}$) and incorporation efficiency (%) using Kahana Bay, Oahu surface water collected May 2006.

| Salinity | Rate (μg C $L^{-1}$ $d^{-1}$, AVG (SD)) | | Incorporation Efficiency (%) |
|---|---|---|---|
| | Incorporation | Mineralization | |
| 0 | 54.8 (12.9) | 0.14 (0.13) | 99.7 |
| 4 | 26.9 (1.37) | 0.52 (0.13) | 98.1 |
| 18 | 20.2 (0.73) | 0.0 (0.0) | 100 |
| 29 | 15.4 (1.18) | 0.39 (0.17) | 97.5 |
| 35 | 3.02 (3.71) | 0.80 (0.76) | 79.1 |

**Table 5.** Average (AVG) rates of TNT incorporation, mineralization (μg C $L^{-1}$ $d^{-1}$) and incorporation efficiency (%) using Kahana Bay, Oahu surface water collected August 2006.

| Salinity | Rate (μg C $L^{-1}$ $d^{-1}$, AVG (SD)) | | Incorporation Efficiency (%) |
|---|---|---|---|
| | Incorporation | Mineralization | |
| 3 | 58.2 (2.9) | 0.43 (0.92) | 99.2 |
| 6 | 38.6 (7.8) | 0.15 (0.88) | 99.6 |
| 10 | 121.2 (13.8) | 0.95 (0.53) | 99.2 |
| 23 | 7.2 (0.97) | 0.0 (0.0) | 100 |
| 35 | 46.3 (15.3) | 0.36 (0.28) | 99.2 |

A third sampling of Kahana Bay was performed during August 2007 and as was the case the previous August sampling (2006), there was little rainfall prior to this salinity survey. In this survery, however, both surface water and sediment were collected. The effect of salinity on TNT

incorporation in the surface water samples was less prominent than in the previous two surveys though the two highest values were at the lowest salinities (Table 6). TNT incorporation rates were about an order of magnitude higher than those for RDX and almost two orders of magnitude higher than those for HMX (Table 6). Mineralization rates for all three NECs were relatively low and did not show a pattern with salinity. Because of background issues, it is difficult to measure bacterial incorporation rates of TNT using sediment so mineralization rates alone were compared for the Kahana Bay survey. When comparing rates on a per kg and per L basis, the values for TNT were very similar in magnitude and pattern with the May '06 surface water survey for TNT incorporation (Table 7) Mineralization rates for $^{14}$C-RDX and $^{14}$C-HMX were also measured for the sediment stations but they showed little pattern with salinity. The highest values for both were at the mid-salinity station (15 PSU).

The metabolism of $^{14}$C-radiolabeled TNT was compared with that of RDX and HMX in water collected from the mouth of the Delaware Bay (34 PSU) in December 2006. The effect of different types of killing agents (TCA, formalin, sulfuric acid) and the incubation of samples in laboratory light verses darkness was determined to be less than 5% (data not shown). Rate of incorporation into bacterial macromolecules was much higher for TNT (35.8 +/- 11.9 μg C $L^{-1}$ $d^{-1}$) than that for RDX or HMX (0.0) (Table 8). Mineralization rate of TNT (0.46 +/- 0.20 μg C $L^{-1}$ $d^{-1}$) was an order of magnitude higher than that of HMX (0.039 +/- 0.002 μg C $L^{-1}$ $d^{-1}$) or RDX (0.014 +/-0.004 μg C $L^{-1}$ $d^{-1}$) though all were low for these surface water samples incubated for 48 hours.

Finally, in the surface waters of the Gulf of Mexico, TNT incorporation rates were one to two orders of magnitude higher than mineralization (Table 9). RDX mineralization was often not detected and incorporation showed little relationship with salinity. HMX incorporation was highest below 30 PSU and often three orders of magnitude higher than corresponding mineralization rates. In offshore sediment, RDX (46.2 +/- 5.78) and HMX (10.44 +/- 0.63) mineralization (μg $kg^{-1}$ $d^{-1}$) were two to four orders of magnitude more rapid than in the surface water samples (kg vs L).

**Table 6**. Average (AVG) rates of TNT, RDX and HMX incorporation, mineralization (μg C $L^{-1}$ $d^{-1}$) using Kahana Bay, Oahu surface water collected August 2007.

| Salinity (psu) | Incorporation Rate (AVG (SD) μg $L^{-1}$ $d^{-1}$) | | | Mineralization Rate (AVG (SD) μg $L^{-1}$ $d^{-1}$) | | |
|---|---|---|---|---|---|---|
| | TNT | RDX | HMX | TNT | RDX | HMX |
| 4 | 35.8 (2.6) | 1.19 (0.63) | 0.33 (0.20) | 0.35 (0.36) | 0.09 (0.28) | 0.65 (0.82) |
| 9 | 40.2 (3.2) | 2.71 (2.75) | 0.00 (0.00) | 3.01 (9.13) | 0.09 (0.45) | 0.24 (1.12) |
| 15 | 24.1 (4.0) | 1.58 (0.54) | 0.42 (0.13) | 1.93 (3.84) | 0.03 (0.38) | 0.76 (0.14) |
| 20 | 26.2 (0.8) | 1.49 (0.70) | 0.89 (0.19) | 0.00 (0.00) | 0.21 (0.23) | 0.72 (0.45) |
| 30 | 27.3 (16.1) | 4.01 (2.16) | 0.75 (1.17) | 0.42 (1.46) | 0.00 (0.00) | 0.67 (0.14) |

**Table 7**. Average (AVG) rates of TNT mineralization (µg C $kg^{-1}$ $d^{-1}$) using Kahana Bay, Oahu sediment collected August 2007.

| Salinity (psu) | Mineralization Rate (AVG (SD) µg $kg^{-1}$ $d^{-1}$) | | |
|---|---|---|---|
| | TNT | RDX | HMX |
| 4 | 50.3 (34.8) | 5.10 (5.12) | 0.00 (0.00) |
| 9 | 48.8 (58.6) | 0.00 (0.00) | 7.28 (9.78) |
| 15 | 30.7 (27.0) | 18.17 (3.98) | 22.96 (11.85) |
| 20 | 14.0 (19.5) | 7.09 (4.62) | 9.79 (9.07) |
| 30 | 8.5 (1.5) | 4.77 (1.35) | 0.00 (0.00) |

**Table 8.** TNT, RDX, HMX incorporation and mineralization rates (µg C $L^{-1}$ $d^{-1}$) were measured on Delaware Bay water (34 PSU) collected in December 2006.

| NEC | Transformation Process | Light Incubation | | | | Dark Incubation | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Whole water | | Pre-Filtered | | Whole water | | Pre-Filtered | |
| | | AVG | SD | AVG | SD | AVG | SD | AVG | SD |
| TNT | Mineralization | 0.46 | 0.20 | 1.73 | 1.17 | 0.41 | 0.13 | 0.46 | 0.03 |
| | Incorporation | 35.8 | 11.9 | 1.01 | 0.17 | 33.9 | 13.1 | 0.93 | 0.12 |
| RDX | Mineralization | 0.014 | 0.004 | 0.0 | | 0.011 | 0.015 | 0.0 | |
| | Incorporation | 0.0 | | 0.007 | 0.003 | 0.008 | 0.007 | 0.0 | |
| HMX | Mineralization | 0.039 | 0.002 | 0.042 | 0.056 | 0.048 | 0.007 | 0.014 | 0.021 |
| | Incorporation | 0.0 | | 0.009 | 0.006 | 0.0 | | 0.006 | 0.039 |

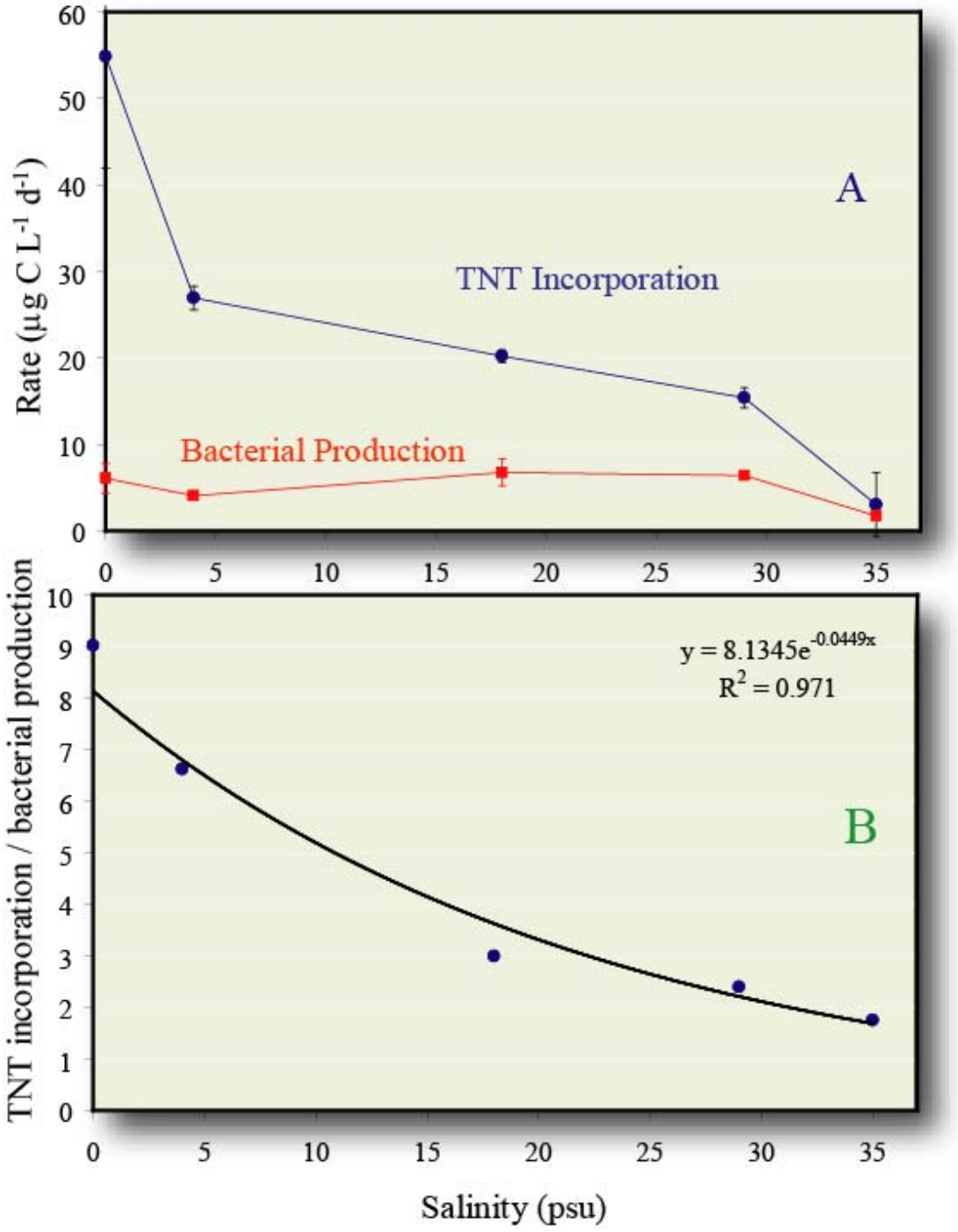


**Figure 9.** For samples of Kahana Bay, Oahu surface water collected May 2006: A) Average (AVG) rates of TNT incorporation and bacterial production (µg C $L^{-1}$ $d^{-1}$); and, B) TNT incorporation normalized for bacterial production and regressed with salinity.

**Table 9.** Incorporation and mineralization rates (μg C $L^{-1}$ $d^{-1}$) for TNT, RDX and HMX were determined for surface water samples taken in the Gulf of Mexico during May 2007. Mineralization rates for one sediment station (μg C $kg^{-1}$ $d^{-1}$) are included for comparison.

| | | TNT | | | | RDX | | | | HMX | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Incorporation | | Mineralization | | Incorporation | | Mineralization | | Incorporation | | Mineralization | |
| | psu | AVG | SD | AVG | SD | AVG | SD | AVG | SD | AVG | SD | AVG | SD |
| water | | | | | | | | | | | | | |
| GOM07010 | 0 | 464 | 501 | 0 | 0 | 0 | 0 | 0 | 0.0 | 171 | 59 | 0 | 0 |
| GOM07009 | 2.5 | 0 | 0 | 0 | 0 | 12 | 20 | 0 | 0.0 | 290 | 86 | 0 | 0 |
| GOM07006 | 4.4 | 0 | 0 | 0 | 0 | 38 | 23 | 0.01 | 0.02 | 593 | 374 | 0.43 | 0.29 |
| GOM07005 | 6.9 | 0 | 0 | 1.09 | 0.54 | 73 | 35 | 0 | 0 | 647 | 151 | 0.14 | 0.10 |
| GOM07017 | 8 | 36 | 20 | | | 0 | 23 | | | 0 | | | |
| GOM07018 | 10 | 11 | 16 | | | 0 | 0 | | | 889 | 871 | | |
| GOM07008 | 12.4 | 0 | 0 | 0.25 | 0.71 | 12 | 15 | 0.04 | 0.03 | 296 | 435 | 0.30 | 0.25 |
| GOM07007 | 20.4 | 35 | 34 | 1.47 | 1.28 | 103 | 66 | 0 | 0 | 384 | 220 | 0 | 0 |
| GOM07004 | 25.3 | 34 | 113 | 1.06 | 1.19 | 0 | 0 | 0 | 0 | 641 | 357 | 0.07 | 0.14 |
| GOM07016 | 30.7 | 13 | 12 | | | 101 | 106 | | | 47 | 76 | | |
| GOM07015 | 30.8 | 0 | 0 | | | 129 | 20 | | | 0 | 0 | | |
| GOM07019 | 32 | 1 | 11 | | | 39 | 179 | | | 0 | 0 | | |
| GOM07003 | 32.2 | 552 | 434 | 2.03 | 0.55 | 57 | 245 | 0 | 0 | 0 | 0 | 0.02 | 0.17 |
| GOM07014 | 33.5 | 26 | 11 | | | 0 | 0 | | | 18 | 46 | | |
| GOM07002 | 35.9 | 0 | 0 | 0 | 0.00 | 32 | 104 | 0 | 0 | 0 | 0 | 0.08 | 0.09 |
| GOM07023-2 | 35.9 | 0 | 0 | | | 767 | 484 | | | 0 | 0 | | |
| GOM07023-4 | 35.9 | 24 | 54 | | | 210 | 197 | | | 0 | 0 | | |
| GOM07023-6 | 35.9 | 263 | 153 | | | 0 | 0 | | | 0 | 0 | | |
| GOM07020 | 36 | 0 | 0 | | | 0 | 0 | | | 0 | 0 | | |
| sediment | | | | | | | | | | | | | |
| GOM07003 | 32.2 | | | 0 | | | | 46.20 | 5.78 | | | 10.44 | 0.63 |

**3.2 Field surveys of NEC degradation rates in water and sediment** Fourteen surveys of coastal ecosystems were conducted from 2002-2008 to measure NEC mineralization rates using standard radiotracer techniques designed to interrogate organic carbon metabolism by natural assemblages (Deming 1992). For these comparisons, the detection limit of our 24 h $^{14}$C-radiotracer incubations with natural sediment samples (1 g wet volume) was 1.0 x $10^{-2}$ μg C $kg^{-1}$ $d^{-1}$ though a sample was only considered to have detectable NEC mineralization rates if the average of the triplicate live values (minus the killed control) was greater than the standard deviation. If the standard deviation was larger than the average mineralization value of the triplicate samples, then that station was listed as a nondetect for TNT mineralization.

In general, TNT mineralization rates by the natural bacterial assemblage in surface sediments were higher than 2,4-DNT and 2,6-DAT mineralization rates measured at the same station. TNT mineralization was detected at 40 out of 56 stations (71%) and ranged from nondetect to 145 (+/- 16.0) μg C $kg^{-1}$ $d^{-1}$ though the median values of the each survey only ranged from 3.0 (+/- 0.56) to 17.6 (+/- 0.49) μg C $kg^{-1}$ $d^{-1}$ (Table 7). These median values may be more useful for determining the capacity of ecosystem sediments to metabolize TNT. Despite the wide variation in ecosystem types (tropical verses temperate), the median TNT mineralization rates were within an order of magnitude among surveys. In addition,

mineralization of 2,4-DNT and 2,6-DAT were also measured in the Chesapeake Bay surveys and the assays were typically conducted at the same stations as those for TNT mineralization. 2,4-DNT and 2,6-DAT mineralization was detected at fewer stations (38% and 58%, respectively) and had a lower median range for stations where mineralization was detected (1.35 +/- 0.44 and 9.88 +/- 0.48 μg C $kg^{-1}$ $d^{-1}$, respectively). In San Francisco Bay, only one survey was performed (May 2003) and TNT mineralization was detected at four out of 10 stations with 2,4-DNT mineralization detected at two out of 10 stations. The Hawaii sampling involved surveys of Bishop's Point and South Loch in Pearl Harbor and Ala Wai Canal in Waikiki with 45% of the samples having detectable TNT mineralization rates (median rate = 3.57 +/- 1.51 μg C $kg^{-1}$ $d^{-1}$). When averaged across all data for a sampling cruise, TNT mineralization rates in the Chesapeake Bay were higher than those for Hawaii and San Francisco Bay.

The bacterial assemblage degradation rate of NEC may be related to metabolism of other aromatic compounds like PAHs ($^{14}$C-naphthalene, -phenanthrene, -fluoranthene), toluene, catechol, or even heterotrophic metabolism in general (bacterial production). Given the wide range of ecosystems and different times of the year of our samplings, we did not find good correlations when comparing TNT mineralization rates with these other parameters on a station-by-station basis. However, we did find that when averaging rates between stations where TNT mineralization was detected verses not detected, the average rates of bacterial production and mineralization of naphthalene, phenanthrene, fluoranthene, catechol and toluene were all higher at stations where TNT mineralization was detected (Table 8). Also, the range in average PAH and toluene mineralization rates for all the stations (0.80 to 8.04 μg C $kg^{-1}$ $d^{-1}$) is very similar to that for TNT (3.08 to 17.6 μg C $kg^{-1}$ $d^{-1}$). This suggests that TNT is mineralized in coastal sediments at rates that are typical for commonly occurring compounds, like phenanthrene and fluoranthene, and even similar to compounds like toluene and naphthalene that are known to be very transient in the estuarine sediments (though appears to be uncoupled with toluene mineralization, Figure 10). Average 2,6-DAT mineralization was higher at stations where TNT mineralization was detected (9.00 verses 3.84 μg C $kg^{-1}$ $d^{-1}$). Somewhat surprisingly, average 2,4-DNT mineralization rate for the TNT mineralization non-detect stations was slightly higher (13.8 verses 10.3 μg C $kg^{-1}$ $d^{-1}$) than for the detect stations suggesting that TNT and 2,4-DNT mineralization may be uncoupled processes amongst the natural assemblage.

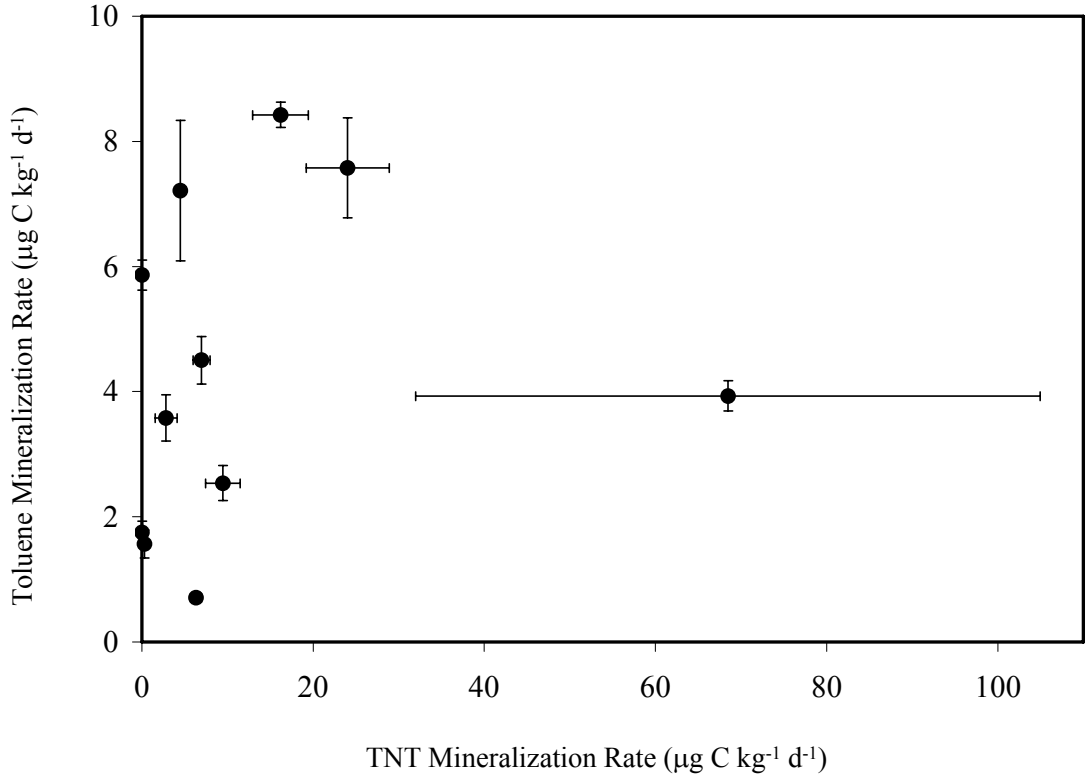


**Figure 10.** Toluene mineralization (µg C $kg^{-1}$ $d^{-1}$) was uncoupled with TNT mineralization in Chesapeake Bay surface sediments in March 2005.

For a Chesapeake Bay sediment sampling in March 2004, rates of TNT mineralization and heterotrophic bacterial production appear to covary for many of the stations (Table 10). The tidal freshwater stations (TIDAL-C, TIDAL-S) are an exception to this as bacterial production is disproportionally higher than TNT mineralization suggesting that TNT is less important as a carbon or nitrogen source to this assemblage. At two stations in the tributaries (Pawtuxet River, PXRS; Rappahannock River, RRC) show the opposite trend of TNT mineralization being disproportionately high relative to bacterial production. These findings gave rise to the hypothesis that salinity regimes affected TNT metabolism by the bacterial assemblage. Specifically, TNT was metabolized more rapidly by the estuarine and marine assemblages (which are nitrogen limited) and much less rapidly by freshwater assemblages (phosphorus limited).

**Table 10**. Surface sediment in the tributaries of the Chesapeake and Delaware bays were sampled in March 2004 and measured for salinity, TNT mineralization, organic carbon (OC, %), bacterial production and the ratio of TNT mineralization to bacterial production (min/prod).

| River | Station | Location | Salinity (psu) | TNT Mineralization (AVG (SD) μg $kg^{-1}$ $d^{-1}$) | OC (%) | Bacterial Production (AVG (SD) μg $kg^{-1}$ $d^{-1}$) | Min/ Prod |
|---|---|---|---|---|---|---|---|
| Susquehanna | TIDAL-C | Channel | 0.2 | 24.3 (3.7) | 3.52 | 327 (88) | 0.07 |
| | TIDAL-S | Shoal | 0.2 | 11.4 (0.4) | 2.53 | 212 (40) | 0.05 |
| Potomac | PRUC | Channel | 5.6 | 146 (16.0) | 2.86 | 251 (15) | 0.58 |
| | PRUS | Shoal | 5.6 | 74.2 (4.0) | 1.52 | 273 (16) | 0.27 |
| | PRMC | Channel | 6.0 | 136 (15.8) | 2.93 | 273 (17) | 0.50 |
| | PRMS | Shoal | 6.7 | 17.6 (0.5) | 0.03 | 41 (28) | 0.43 |
| Delaware | DRC | Channel | 10.2 | 10.4 (4.8) | 1.52 | 20 (6) | 0.52 |
| | DRS | Shoal | 12.3 | 4.2 (1.3) | 0.99 | 13 (2) | 0.31 |
| Rappahannock | RRC | Channel | 11.4 | 58.2 (4.2) | 1.70 | 39 (20) | 1.47 |
| | RRS | Shoal | 11.2 | 9.8 (0.5) | 0.05 | 56 (15) | 0.17 |
| Pawtuxet | PXRC | Channel | 11.9 | 5.0 (0.9) | 2.22 | 120 (13) | 0.04 |
| | PXRS | Shoal | 10.7 | 103 (3.8) | 0.16 | 25 (6) | 4.16 |
| Elizabeth | ERC | Channel | 13.0 | 93.9 (11.8) | 1.50 | 115 (6) | 0.81 |
| | ERS | Shoal | 13.0 | 34.0 (6.5) | 0.86 | 87 (6) | 0.39 |
| James | JRC | Channel | 13.6 | 13.8 (3.5) | 0.42 | 27 (8) | 0.51 |
| | JRS | Shoal | 13.6 | 8.9 (2.3) | 0.14 | 42 (8) | 0.21 |
| York | YRC | Channel | 15.3 | 12.8 (2.6) | 0.42 | 31 (21) | 0.41 |
| | YRS | Shoal | 15.3 | 57.5 (19.9) | 2.08 | 190 (33) | 0.30 |

During a March 2005 sediment survey of the Chesapeake Bay watershed, TNT mineralization relative to production was again lower in the lower salinity portion of the transect (Table 11). TNT mineralization was highest relative to bacterial production at the mouths of the Pawtuxet and Rappahannock Rivers, as it was in the March 2004 survey.

**Table 11**. Surface sediment in the tributaries of the Chesapeake and Delaware bays were sampled in March 2005 and measured for salinity, TNT mineralization/production, amino acid metabolism, and heterotrophic bacterial production.

| Station | Salinity | TNT Mineralization /Production AVG (SD) | Amino Acid Metabolism µg C $L^{-1}$ $d^{-1}$ | Heterotrophic Production µg C $kg^{-1}$ $d^{-1}$ |
|---|---|---|---|---|
| TPF | 0.12 | 0.28 (0.13) | | 10.11 (1.0) |
| DBF | 5.82 | 0.00 (0.00) | 18.90 | 3.66 (0.33) |
| PE1 | 11.91 | 0.26 (0.05) | 2.00 | 61.5 (24.3) |
| ERC | 12.00 | 0.26 (0.04) | | 26.8 (2.30) |
| GWR | 12.16 | 0.00 (0.00) | 3.23 | 6.07 (0.79) |
| PAX | 12.20 | 14.6 (7.7) | | 4.74 (0.17) |
| RAP | 13.09 | 1.33 (0.27) | 0.61 | 18.1 (2.11) |
| YRC | 15.01 | 1.41 (0.30) | | 6.71 (0.15) |
| YR1 | 16.35 | 1.60 (0.16) | 13.97 | 3.96 (0.30) |
| JRC | 17.07 | 0.59 (0.05) | 28.10 | 7.63 (1.73) |
| BYM | 29.87 | 0.04 (0.02) | 0.07 | 7.09 (2.27) |

As part of the March 2004 Chesapeake Bay survey, dissolved organic carbon (DOC) concentration and composition was examined across the salinity transect. DOC is produced in the lower salinity waters of the tributaries (Figure 11B). The DOC appears to be terrestrial based on its stable carbon isotope signature (Figure 11C) and at the Pawtuxet River mouth, it contains high concentrations of lignin (Figure 11D). The DOC decreases rapidly over salinity range of 13 to 20 PSU, which is also where the highest TNT mineralization rates relative to production occur. Based on literature reports (Harman-Fetcho et al 1999, McConnell et al 2004 and references therein), the Patuxent River mouth area appears to receive large concentrations of agricultural runoff including lignocellulose and nitrogenous pesticides, including simazine (Figure 11-lower right inset). The sediment here is also associated with bacterial assemblages that rapidly metabolize nitrogenous pesticides (McConnell et al 2004).

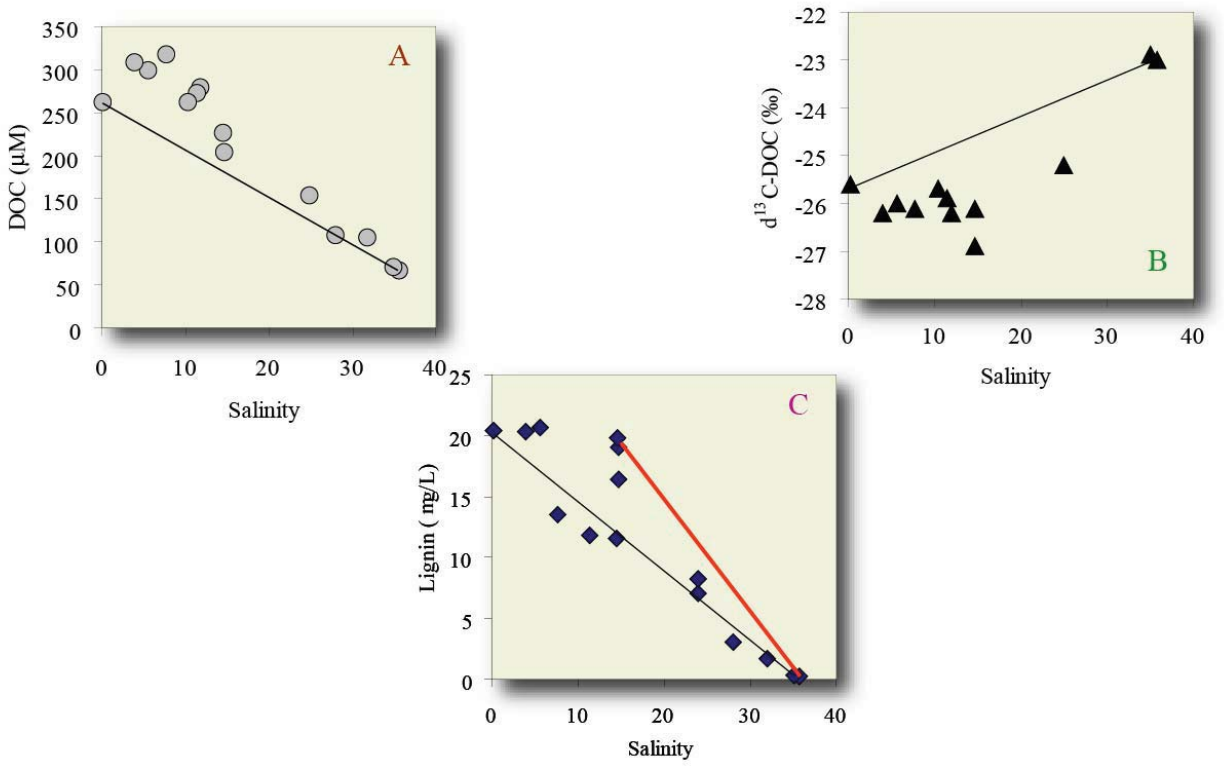


**Figure 11.** Chesapeake Bay stations were sampled in March 2004. A) Dissolved organic carbon (DOC) is produced in low salinity tributaries in the upper bay; B) This DOC appears to be terrestrial rather that phytoplankton derived based on its stable carbon isotope values in the upper bay; C) DOC appears to be high in lignin concentration, especially in the Patuxent River.

It is possible that the Chesapeake Bay transect can be the result of the three TNT transformation processes originally described in the literature. In the freshwater end-member, TNT is metabolized dissimilatorily and not as a carbon or nitrogen source. The high incorporation efficiencies may be an artifact of binding of aminotoluenes to particulate matter (or even the bacteria themselves). The low TNT mineralization rates measured are consistent with this hypothesis. Some areas of exceptionally high mineralization rates may be the result of rapid lignin metabolism. These areas include the Patuxent River mouth and the zones of convergence between water masses in Kahana Bay. Bacterial assemblages in much of the estuarine and marine ecosystems may be assimilating TNT, similarly to that of other common organic nitrogen source. This assimilatory use of TNT may be highly efficient as it is for other organic nitrogen source like amino acids, or it may be less efficient as it is with chitin. Note that the mineralization rates for TNT are similar to those reported for chitin in the Delaware Bay (0.41-2.45 µg C $L^{-1}$ $d^{-1}$; Kirchman and White 1999) and TNT incorporation rates are similar to those of amino acids (Table 2 verses Table 11). This would explain the covariance between TNT mineralization and heterotrophic production (which is measured by amino acid incorporation). If TNT is preferentially incorporated into bacterial proteins rather than catabolized for energy, as are amino acids, then the mineralization rates measured may actually be remineralization of bacterial macromolecules by protozoan grazers. Remineralization rates would likely correlate with bacterial production rates needed to support growth of the protozoan grazer population.

**Table 12.** Nitrogenous energetic compound (NEC) mineralization rates (μg C $kg^{-1}$ $d^{-1}$) were surveyed for surface sediments in three coastal ecosystems from 2002 to 2005. The range and median rate are reported for the collection of samples in each ecosystem where mineralization was detected (sample AVG: average; SD: one standard deviation).

| Ecosystem (# sampling events) | NEC | Total Stations | Detects | Mineralization Range AVG μg C $kg^{-1}$ $d^{-1}$ (+/-SD) | | Median Rate for Detects AVG (+/-SD) |
|---|---|---|---|---|---|---|
| | | | | Low | High | |
| Chesapeake Bay (3) | TNT | 35 | 31 | 0.29 (+/-0.13) | 145 (+/-16.0) | 17.6 (+/-0.49) |
| | DNT | 26 | 10 | 0.14 (+/-0.05) | 219 (+-119) | 1.35 (+/-0.44) |
| | DAT | 17 | 10 | 2.15 (+/-0.30) | 49.3 (+/-22.6) | 9.88 (+/-0.48) |
| San Francisco Bay (1) | TNT | 10 | 4 | 2.96 (+/-1.33) | 7.42 (+/-2.11) | 3.08 (+/-0.56) |
| | DNT | 12 | 2 | 16.0 (+/-12.4) | 68.2 (+/-12.8) | Not Applicable |
| Hawaii (2) | TNT | 11 | 5 | 2.38 (+/-1.60) | 19.9 (+/-11.8) | 3.47 (+/-1.51) |

Evidence from the literature suggests that both aerobic and anaerobic conditions may be required for complete TNT mineralization though most of this information is derived from culture work and laboratory conditions. Nonetheless, we examined bacterial TNT mineralization rates in core sections (typically 2-4 cm) down to 20 cm below the water-sediment boundary. Gravity cores were sectioned and then one mL wet volume subsamples of sediment were assayed. These cores were taken from Pearl Harbor (Bishop's Point, South Loch), the Ala Wai Canal, the lower Chesapeake Bay (including the York and Elizabeth Rivers), and San Francisco Bay (Alameda, Treasure Island, Hunter's Point). Many of these cores were bioturbated to various degrees in the upper sections or throughout.

As with the previous survey of surface sediments, the core section data were grouped based on whether or not TNT mineralization was detected or not and then compared with other metabolic analyses. In December 2002, four cores were taken at Bishop's Point and South Loch (2 each) in Pearl Harbor, HI. TNT mineralization rates were detected in 6 out of 22 core sections of the 4 cores. Bacterial production averaged among the sections where TNT mineralization was detected was only slightly higher than among those sections where mineralization was not detected (6.50 verses 6.21 μg C $kg^{-1}$ $d^{-1}$; Table 13). In the comparison of the surface sediments described above, the difference between the TNT mineralization detects and nondetects was about seven-fold greater with respect to production (Table 14). Average mineralization rates of the PAHs, naphthalene, phenanthrene, and fluoranthene, were all higher for the core sections where TNT mineralization was detected as was seen in the surveys of the surface sediments from the three ecosystems (Table 14).

**Table 13.** Average (AVG) bacterial production and mineralization rates (μg C $kg^{-1}$ $d^{-1}$) were measured on sections from four sediment cores taken from Pearl Harbor, HI in December 2002.

Although production was similar when averaged among sections where TNT mineralization was not detected relative to sections where it was detected, PAH mineralization rates were higher among the latter sections.

| TNT Mineralization Rate | AVG Production or Mineralization Rate (µg C $kg^{-1}$ $d^{-1}$) | | | |
|---|---|---|---|---|
| | Bacterial production | Naphthalene | Phenanthrene | Fluoranthene |
| Detects (6) | 6.50 | 0.20 | 5.41 | 1.83 |
| Non-detects (16) | 6.21 | 0.05 | 2.31 | 0.02 |

**Table 14.** TNT mineralization was measured on sediment samples (68 total) from the Chesapeake Bay (35), San Francisco (10) and the Ala Wai canal, HI (11). Stations with detectable TNT mineralization rates were compared with those that were below the detection limit. Average (AVG) bacterial production and mineralization rates (µg C $kg^{-1}$ $d^{-1}$) for other organic compounds were generally higher for samples that had measurable TNT mineralization with the notable exception of DNT.

| TNT Mineralization Rate | Production or Mineralization Rate (µg C $kg^{-1}$ $d^{-1}$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Bacterial production | Naphthalene | Phenanthrene | Fluoranthene | Catechol | DNT | DAT | Toluene |
| Detects (42) | 76.3 | 4.79 | 8.04 | 1.06 | 160 | 10.3 | 9.00 | 4.45 |
| Non-detects (26) | 10.1 | 1.40 | 4.66 | 0.80 | 113 | 13.8 | 3.84 | 3.81 |

The ratio of TNT mineralization rate to bacterial production was determined for same core sections and compared the ranges in these values between the surface section (0-2 cm) and below the surface section (2-20 cm). Bacterial production, as measured by the leucine incorporation method, often decreases dramatically with depth in gravity core sections. A high ratio indicates that TNT mineralization could comprise a large component of total bacterial production or that a relatively larger fraction of the natural assemblage could metabolize TNT. For the San Francisco Bay cores (which were heavily bioturbated), there was little difference in the range between the ratios in the surface verses deeper in the sediments (Table 15). However, there was a higher range of ratios between the surface verses the deep sections in both the cores from Chesapeake Bay (surface: 0.04-14; deep: 2.87-40.4) and Hawaii cores (surface: 0.29-0.66; deep: 1.18-26.3). This suggests that deeper sediments may harbor a natural bacterial assemblage that may be better adapted for mineralizing TNT or similarly metabolized types of organic carbon.

**Table 15.** TNT mineralization rate (μg C $kg^{-1}$ $d^{-1}$) as a function of bacterial production (μg C $kg^{-1}$ $d^{-1}$) was determined as a relative measure of the ability of a bacterial assemblage to metabolize TNT for core segments from San Francisco and Chesapeake Bays and Hawaii.

| Ecosystem | Sample Depth (cm below sediment-water interface) | TNT Mineralization Rate/ Bacterial Production (range) | |
|---|---|---|---|
| | | Low | High |
| San Francisco Bay | 0-2 | 0.05 | 1.05 |
| | 2-20 | 0.05 | 0.98 |
| Chesapeake Bay | 0-2 | 0.04 | 14.0 |
| | 2-20 | 2.87 | 40.4 |
| Hawaii | 0-2 | 0.29 | 0.66 |
| | 2-20 | 1.18 | 26.3 |

Gravity cores were taken from three stations in the Chesapeake Bay system for comparison of bacterial production and mineralization of PAHs and TNT: LY, a relatively bioturbated station in the York River (Figure 12); M, a relatively unmixed and armored station in the mid bay (Figure 13); and POD, a physically mixed station in the lower bay (Figure 14) (Arzayus et al. 2001). TNT mineralization with depth was similar to, or higher than, PAH mineralization at the same depth in the Chesapeake Bay cores. The most difference between PAH and TNT mineralization was at the physically mixed station (Figure 14). The more bioturbated station, LY, had the most sections with detectable PAH and TNT mineralization rates (Figure 12).

Gravity cores were also taken from three stations in the San Francisco Bay system for comparison of bacterial production and PAH and TNT mineralization: Alameda (Figure 15), Hunter's Point (Figure 16), and Treasure Island (Figure 17) were cored. Although all cores showed evidence of bioturbation (presence of burrowing worms), the Treasure Island core was characterized by large worms (>20 cm) and light, hydrated sediments. Here the gravity core penetrated 170 cm whereas the other cores were only 20 cm at Alameda and Hunter's Point. Unlike other systems (Figure 12), bacterial production was not as disproportionately elevated in the uppermost sections of the three San Francisco Bay core stations (Figures 15A, 16A, 17A). At all three stations, TNT mineralization was often higher than that for naphthalene and fluoranthene but lower than phenanthrene mineralization when compared by each depth section (Figures 15B, 15C, 16B, 16C, 17B, 17C).

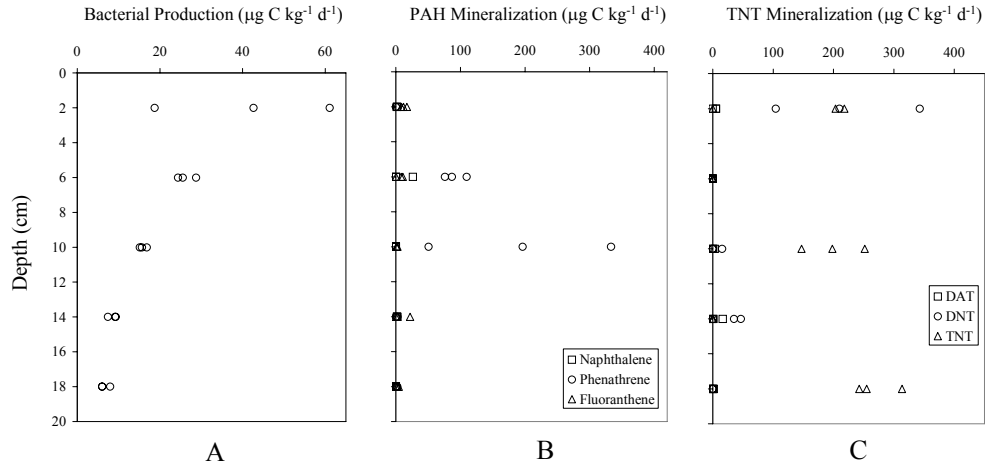


**Figure 12.** Bacterial production (A), PAH mineralization (B) and TNT mineralization (C, µg C kg$^{-1}$ d$^{-1}$) was measured with depth on a sediment core taken from the bioturbated lower Chesapeake Bay station LY in the York River in September 2002.

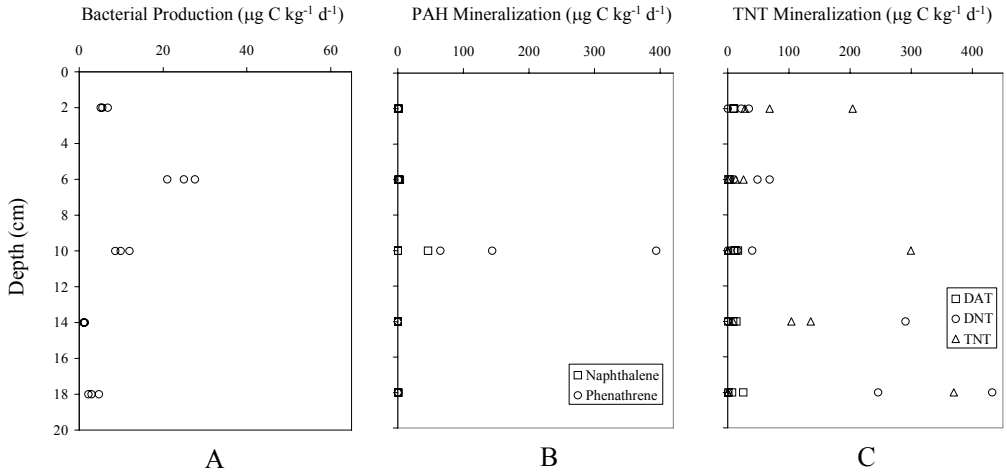


**Figure 13.** Bacterial production (A), PAH mineralization (B) and TNT mineralization (C, µg C kg$^{-1}$ d$^{-1}$) was measured with depth on a sediment core taken from the armored mid Chesapeake Bay station M3 in September 2002.

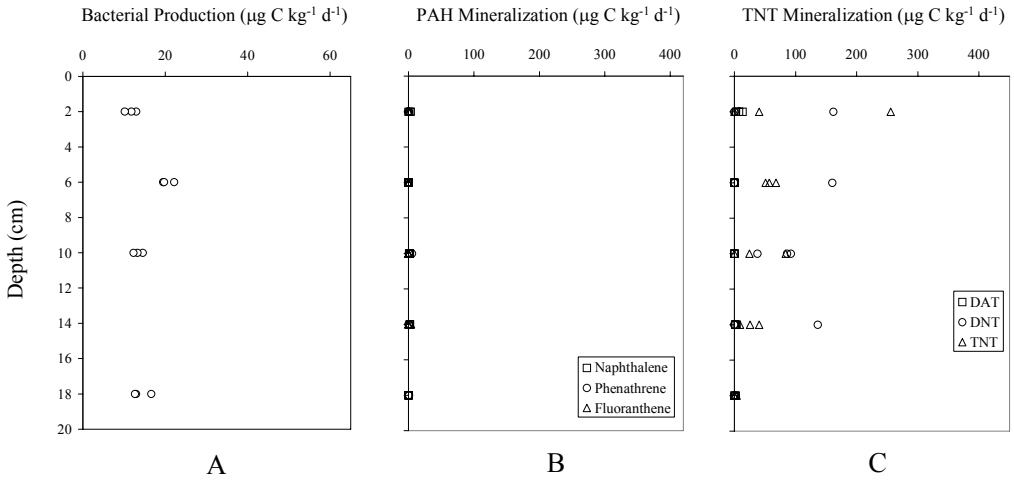


**Figure 14.** Bacterial production (A), PAH mineralization (B) and TNT mineralization (C, µg C kg$^{-1}$ d$^{-1}$) was measured with depth on a sediment core taken from the physically mixed lower Chesapeake Bay station POD in September 2002.

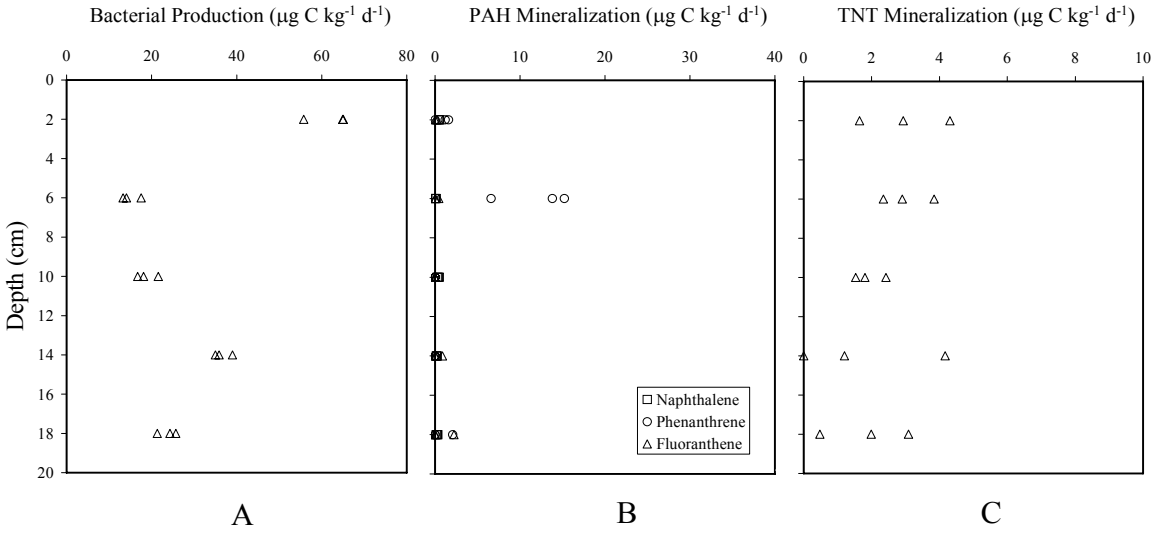


**Figure 15.** Bacterial production (A), PAH mineralization (B) and TNT mineralization (C, µg C kg$^{-1}$ d$^{-1}$) was measured with depth on a sediment core taken off of Alameda in San Francisco Bay in May 2004.

**3.3 Sample seawater during and after subsurface detonation** TNT and RDX are used for underwater demolition in construction and to remove hazards such as unexploded ordnance (UXO). In May 2005, we sampled seawater and sediment at two offshore stations where underwater demolition was being performed in separate detonations of 10 lbs of TNT and 10 lbs of RDX. We sampled prior to the detonation and then immediately afterwards (within 5 min) in the debris field and then followed the plume sampling every 15 min until it could no longer be seen from the surface (ca. 1 h). We then used the surface water samples collected pre-detonation and immediately post-detonation in shoreside incubations in ambient laboratory light and maintained at *in situ* temperature (27$^{o}$C) to determine the degradation rates of the residual

TNT and RDX that were expected to result from the incomplete combustion of the detonation charges.

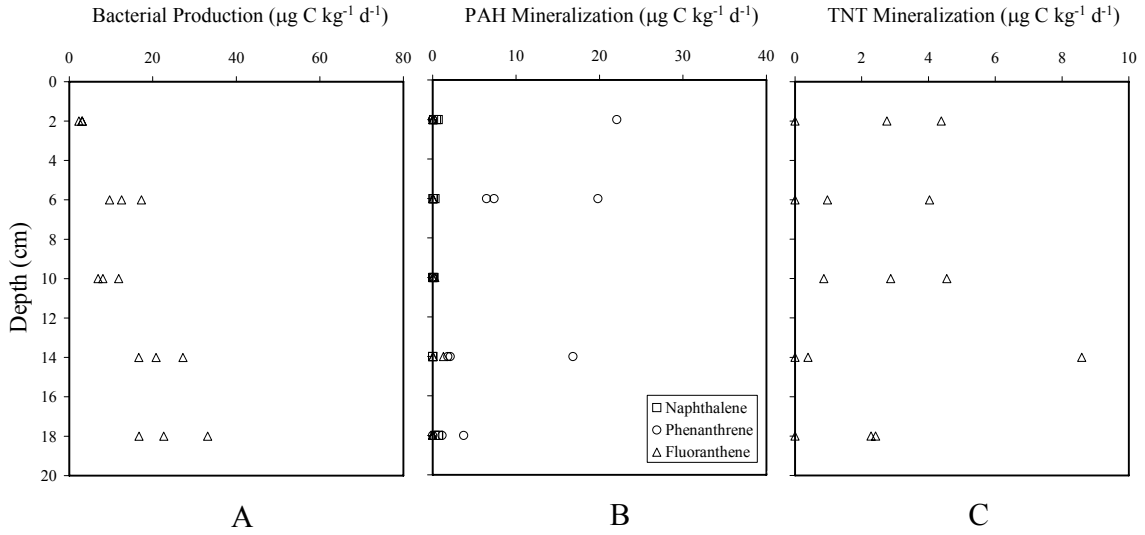


**Figure 16.** Bacterial production (A), PAH mineralization (B) and TNT mineralization (C, µg C $kg^{-1}$ $d^{-1}$) was measured with depth on a sediment core taken off of Hunter's Point in San Francisco Bay in May 2004.

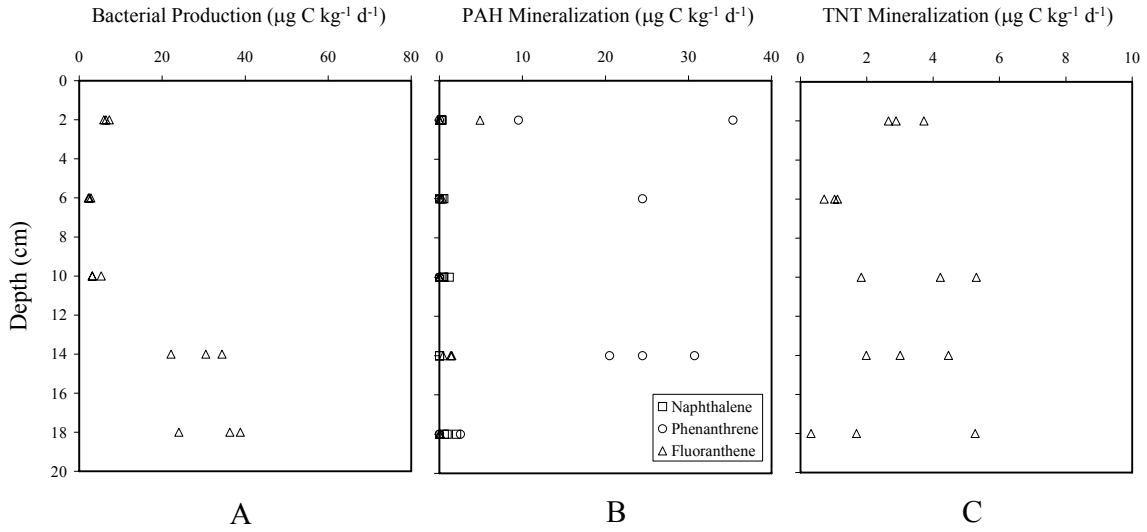


**Figure 17.** Bacterial production (A), PAH mineralization (B) and TNT mineralization (C; µg C $kg^{-1}$ $d^{-1}$) was measured with depth on a sediment core taken off of Treasure Island in San Francisco Bay in May 2004.

Residual TNT and RDX were below the detection limit in seawater (LCMS: 0.6 ppm) even in the samples collected in the plume within 5 minutes after the detonation. Therefore, we were unable to determine rates of photolysis and biodegradation of the energetics in the

shoreside incubations within the plume samples. However, PUV light profiles were taken at the stations prior to the respective detonations, as well as, throughout the plume immediately after detonation and every 10 min following the migration of the plume until it dissipated (Figure 18). They appear to show the dissipation of the effects of the detonation on the water column light penetration as the plume migrates, mixes with seawater outside the plume, and as particles fall out of the water column.

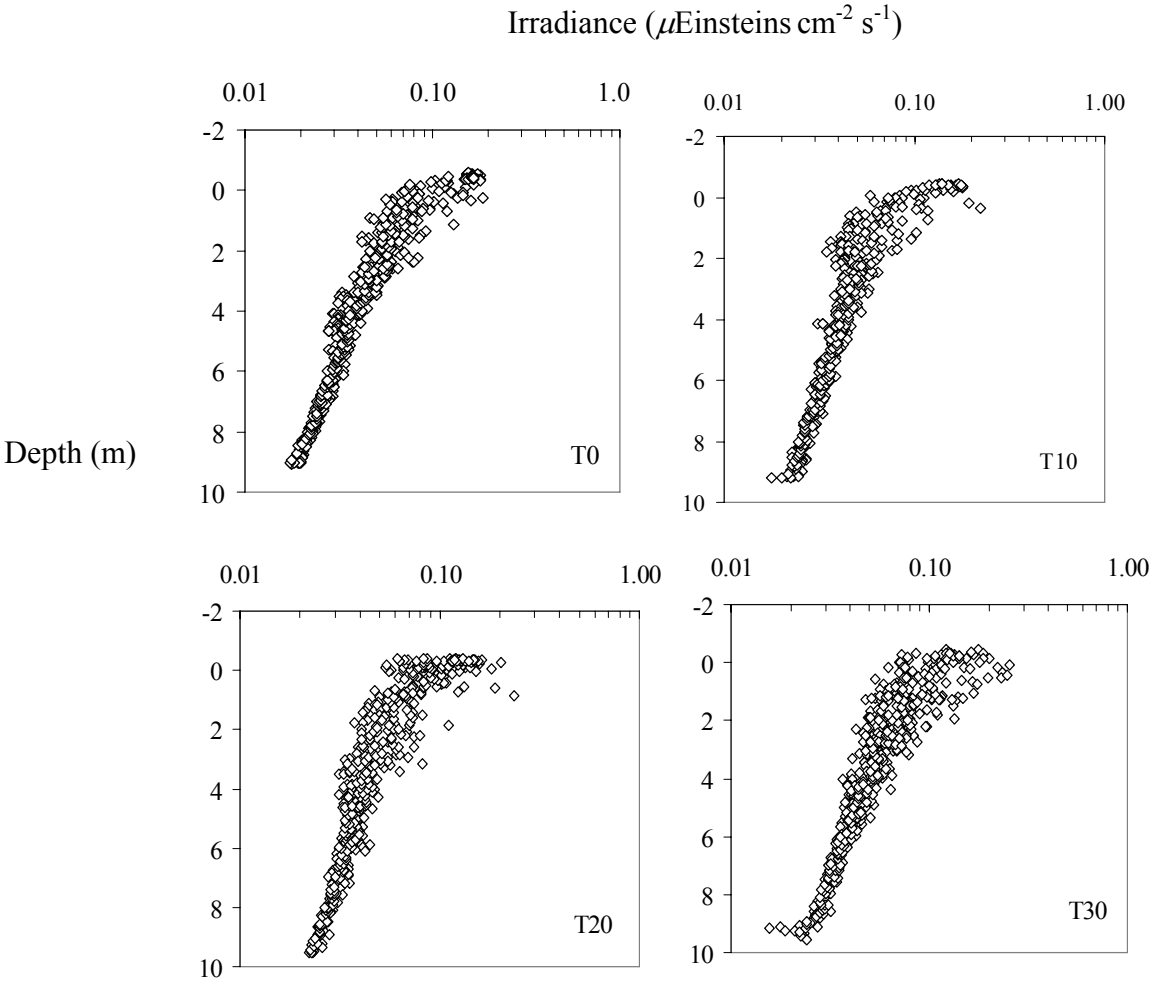


**Figure 18.** Integrated visible light (400-700 nm) profiles offshore of Oahu in May 2005.

Even though there were no detectable NECs in the plume samples, there appeared to be some effect of the detonations on the natural microbial assemblage. Bacterial production appeared to be unchanged following the TNT detonation in surface seawater taken pre versus post-detonation whereas there was an increase in bacterial production in the plume related to the RDX detonation (Table 16). Even though we were unable to detect NEC in the plume seawater samples, it is possible that RDX decomposition resulting from detonation could have resulted in a nutrient release (i.e. nitrate) which could stimulate heterotrophic bacterial metabolism. The postulated nutrient release could be due to simple mixing of the surface sediment with the water

column. TNT uptake and mineralization was measured using radiotracer addition to the subsamples of the incubations. Light appeared to increase uptake of TNT in roughly the same relative amount that light increased bacterial production (Table 17). There was no obvious change in bacterial assemblage composition over the 48 h of the incubations, as determined by DGGE profiles of extracted DNA (data not shown).

**Table 16.** Pacific Ocean seawater was sampled pre and post an underwater demolition detonation with 10 lbs. of TNT or RDX. There was little difference in bacterial production (μg C $L^{-1}$ $d^{-1}$) because of the detonation of TNT, but production roughly doubled in the post RDX detonation sample incubated in either ambient light or dark.

| Energetic | Illumination | Detonation | Bacterial Production (μg C $L^{-1}$ $d^{-1}$) | |
|---|---|---|---|---|
| | | | AVG | SD |
| TNT | Light | Pre | 3.36 | 0.03 |
| | | Post | 3.33 | 0.37 |
| | Dark | Pre | 2.06 | 0.28 |
| | | Post | 1.73 | 0.63 |
| RDX | Light | Pre | 1.83 | 0.30 |
| | | Post | 4.77 | 1.42 |
| | Dark | Pre | 1.27 | 0.79 |
| | | Post | 2.32 | 0.19 |

**Table 17.** Pacific Ocean seawater was sample pre and post an underwater demolition detonation with 10 lbs. of TNT. There was little difference in bacterial production, TNT mineralization or uptake rate (μg C $L^{-1}$ $d^{-1}$) as a result of the detonation (AVG: average; SD, one standard deviation). Incubation under ambient laboratory light increased both bacterial production and TNT uptake (μg C $L^{-1}$ $d^{-1}$); mineralization was not measured under dark conditions. Added 0.176 ppm of TNT.

| Illumination | Detonation | Bacterial Production (μg C $L^{-1}$ $d^{-1}$) | | TNT Uptake (μg C $L^{-1}$ $d^{-1}$) | TNT Mineralization (μg C $L^{-1}$ $d^{-1}$) | |
|---|---|---|---|---|---|---|
| | | AVG | SD | Range | AVG | SD |
| Light | Pre | 3.36 | 0.03 | 161-170 | 5.00 | 2.55 |
| | Post | 3.33 | 0.37 | 231-241 | 4.99 | 2.39 |
| Dark | Pre | 2.06 | 0.28 | 71-123 | --- | --- |
| | Post | 1.73 | 0.63 | 88-100 | --- | --- |

At T = 0, primary production rates in the light and dark plume samples were not significantly different ($P = 0.05$) from their respective controls (Figure 19). This was the case for dark samples for the duration of the experiments. At T = 24 h, particulate primary production (PPP) in the light RDX plume sample and the light TNT plume sample were significantly higher than their respective controls. PPP in the light RDX plume sample continued to rise to the end of the experiment, but decreased slightly in the RDX control. Conversely, PPP in the light TNT plume sample was significantly elevated compared to the control at T = 24 h, but returned to control levels by T = 48 h. It is important to note that PPP in light samples collected from the TNT plume site at T = 0 h was almost double the values from the RDX plume site Also, the absolute difference between the TNT control and plume sample at T = 24 h was greater than the RDX control and plume at the same time. These data indicate that although RDX had a more lasting effect on increasing primary productivity than TNT this may be related to a slower rate of uptake of the RDX daughter products resulting from the detonation by marine algae. It appears that the transformation products (resulting from detonation) of TNT were more labile to phytoplankton and hence were utilized rapidly and exhausted by the end of the experiment. None of the treatments (light vs. dark, plume vs. control) resulted in a significant difference in the rate of phytoplankton production of dissolved organic matter (DPP). DPP production was not statistically different from zero across treatments and for samples collected from both sites for the duration of the experiment. The introduction of the post detonation products of RDX and TNT appear only to affect the production of particulate biomass, and not the production of dissolved photosynthate.

Differences in bacterial production among treatments appeared more related to changes in primary production rather than anything specific to the detonations. The TNT detonation did not appear to impact primary production or bacterial production. This suggests that the stimulation of microbial growth due to the RDX detonation may not have been the result of simple mixing of the sediment (and associated nutrients) and the overlying water column.

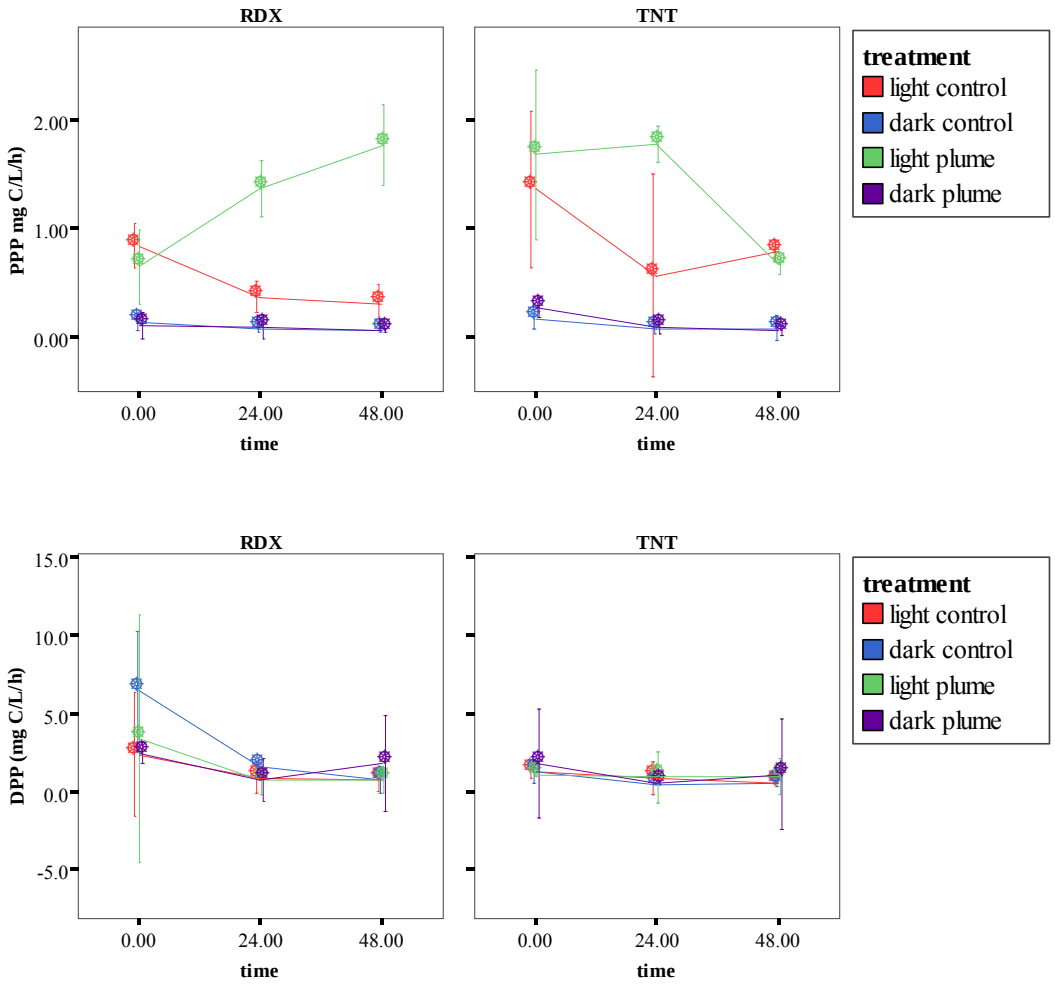


**Figure 19.** Primary production surface water sampled offshore of Oahu in May 2005.

**4.0 Conclusions** Our original hypothesis was that nitrogenous energetics (like TNT, HMX and RDX) would be transient in coastal ecosystems. This was based primarily on the understanding that microbial growth in these systems is typically nitrogen limited and there are few examples of nitrogen based organic compounds that are not rapidly metabolized in these systems. We segregated the TNT degradation literature into three categories based on the importance of organic nitrogen to the transformation process (Figure 20):

NITROGEN (N) INDEPENDENT includes abiotic processes like photolysis and chemical hydrolysis where the presence of energetics (as a nitrogen source for microbiota) would have little impact. Research papers involving abiotic (N independent) processes most often reported the production of reduced products from TNT transformation (e.g. aminotoluenes) (Larson et al 2008). These products would bind to humic particles and aggregates (if present) or

form dimers in solution. Some papers report the photolytic mineralization of TNT to $CO_2$ (Wani et al 2006, Yardin and Chiron 2006).

NOT N LIMITED includes most laboratory culture conditions, freshwater and groundwater environments, soils and some highly contaminated sediment where N species would be the subject of dissimilatory processes for energy production (e.g. reduction of nitrate to ammonia). The reported products for systems that were not N limited were very similar to those for abiotic processes (e.g. aminotoluene) (Cruz-Uribe and Rorrer 2005, Gonzalez-Perez et al 2007, Newcombe and Crawford 2005, 2007, Nyanhongo et al 2006, Popesku et al 2006, Smets et al 2007). The amount of mineralization (usually reported as a percentage of the starting material rather than a rate) is usually low (less than 2%).

N LIMITED includes most marine and estuarine systems where organic nitrogen would be the subject of assimilatory processes for new biomass production (e.g. proteins synthesis). The literature demonstrating that lignolytic fungi can mineralize substantial amounts TNT to $CO_2$ is well established. The exoenzymes that are used for lignin degradation are known to degrade many aromatic organic contaminants and produce ring cleavage products from TNT. The fungi (or lignolytic bacteria) can then take up the degradation products and either catabolize them for energy (mineralize) or incorporate the products into new biomass (Van Aken et al 1999a,b). There are no reports of TNT metabolism by marine or estuarine microbial assemblages outside of this project, though there is for cultured bacteria (Tront and Hughes 2005) and mixed assemblages in the laboratory (Robertson and Jjemba 2005). Typically, organic nitrogen is incorporated into heterotrophic bacterial biomass with catabolism and mineralization occurring only under unusual circumstances (carbon limitation). However, it would be expected that metabolism of bacteria by protozoan grazers would result in the mineralization of some proportion of the bacterial macromolecules.

During 14 sampling events in coastal waterways from 2002 to 2007, we measured TNT mineralization rates in surface sediment and water samples that were often the same as, or within one order of magnitude of, the rate of total heterotrophic bacterial metabolism. These rates were often similar to those of other organic compounds that are transient in natural ecosystems due to their use in bacterial metabolism, such as petroleum hydrocarbons and amino acids. These findings appear to conflict with those that are widely reports in the literature based on the study of cultured microbial strains and groundwater systems. However, the apparent incongruity may be more associated with the specific system studied and the nature of predominant metabolic system of the microbial assemblage.

For a Chesapeake Bay sampling in March 2004, rates of TNT mineralization and heterotrophic bacterial production appear to covary for many of the stations (Figure 21). The tidal freshwater stations (TIDAL-C, TIDAL-S) are an exception to this as bacterial production is disproportionally higher than TNT mineralization suggesting that it is less important as a carbon or nitrogen source to this assemblage. At two stations in the tributaries (Pawtuxet River, PXRS; Rappahannock River, RRC) show the opposite trend of TNT mineralization being disproportionately high relative to bacterial production. These findings gave rise to the hypothesis that salinity regimes affected TNT metabolism by the bacterial assemblage. Specifically, TNT was metabolized more rapidly by the estuarine and marine assemblages

(which are nitrogen limited) and much less rapidly by freshwater assemblages (phosphorus limited).

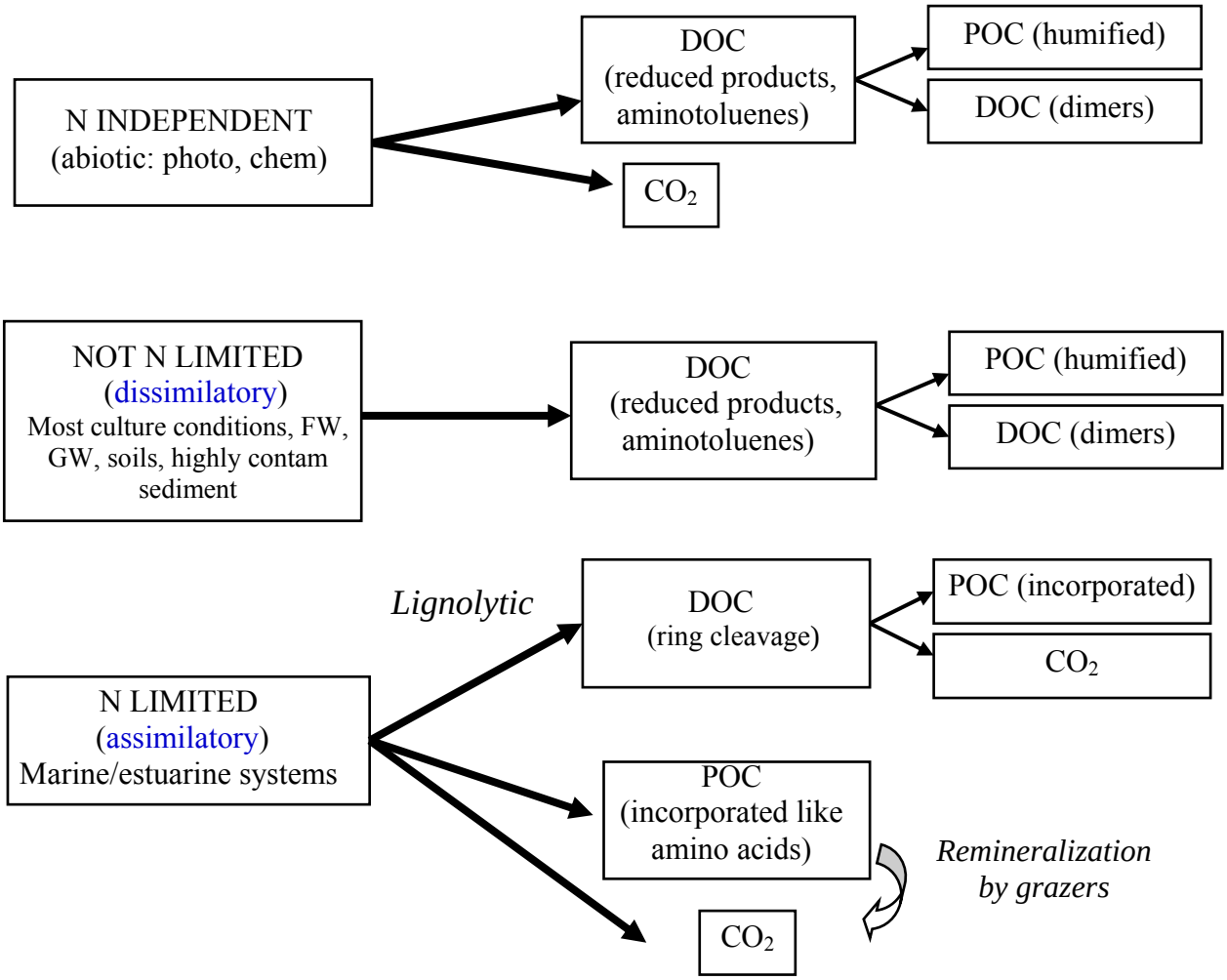


**Figure 20.** Production of dissolved organic carbon (DOC), particulate organic carbon (POC), or carbon dioxide can result from TNT transformation or metabolism depending on whether or not the process is affected by the presence or absense of nitrogen.

Using another measure of TNT metabolism (incorporation of TNT carbon into bacterial biomass) during a Kahana Bay salinity transect of bottom water, it appeared that there was an effect of salinity on bacterial TNT metabolism but that it was the opposite of the original hypothesis. In this well flushed, small estuary, TNT was incorporated most rapidly by the freshwater assemblage (Figure 9A). In addition, the trend was not as robust during subsequent samplings of this transect (August 2006, May 2007) as the flow likely decreased due to reduced rainfall prior to the samplings. The 10 PSU station that had the highest incorporation rate of any station occurred at a boundary between water mass (convergent zone) (Table 5). These transition zones typically collect large particles and wood debris and are the site of rapid organic matter processing.

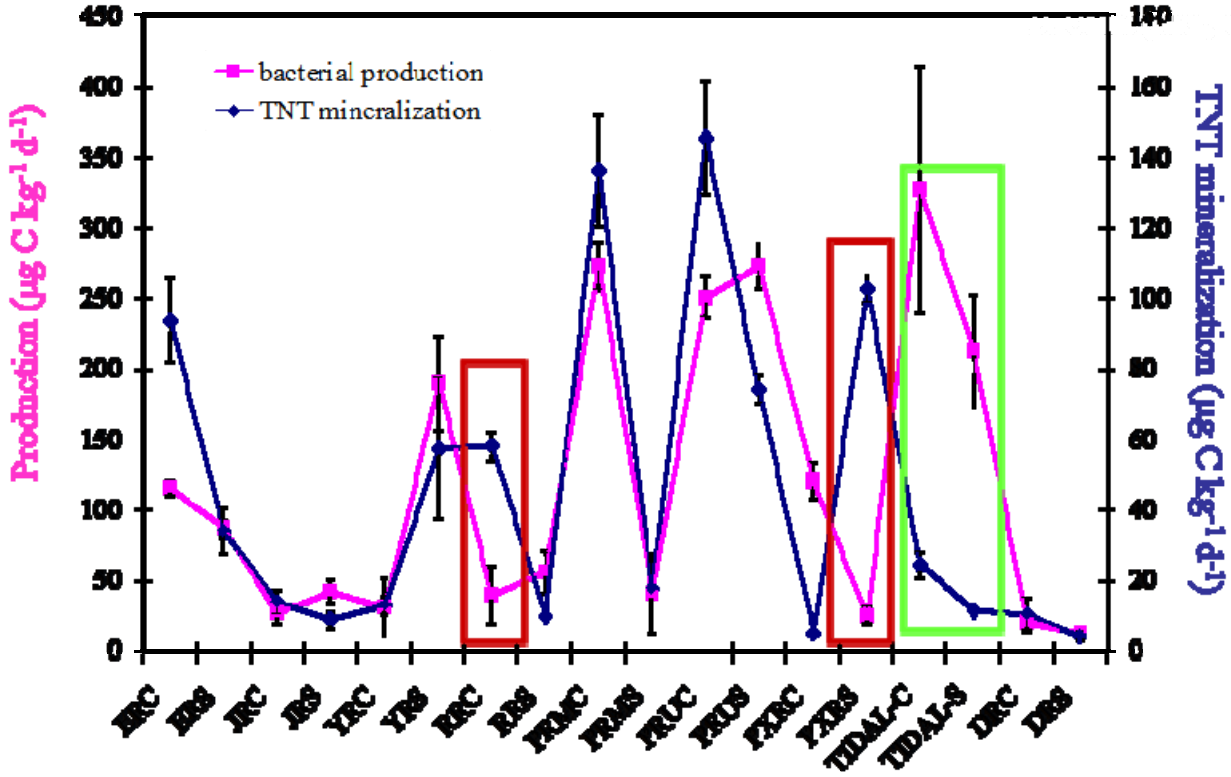


**Figure 21.** Bacterial production and TNT mineralization over a Chesapeake Bay sediment sampling (March 2004). Stations with relatively high (red boxes) and low (green box) TNT mineralization are highlighted.

During a March 2005 sediment survey of the Chesapeake Bay watershed, TNT mineralization (as opposed to incorporation) relative to production was again lower in the lower salinity portion of the transect. TNT mineralization was highest relative to bacterial production at the mouths of the Pawtuxet and Rappahannock Rivers, as it was in the March 2004 survey. As part of the March 2004 Chesapeake Bay survey, dissolved organic carbon (DOC) concentration and composition was examined across the salinity transect. DOC is produced in the lower salinity waters of the tributaries (Figure 11B). The DOC appears to be terrestrial based on its stable carbon isotope signature (Figure 11C) and at the Pawtuxet River mouth, it contains high concentrations of lignin (Figure 11D). The DOC also appears to be removed from the water column over salinity range of 13 to 20 PSU, which is also where the highest TNT mineralization rates relative to production occur. Based on literature reports (Harman-Fetcho et al 1999, McConnell et al 2004 and references therein), the Patuxent River mouth area appears to receive large concentrations of agricultural runoff including lignocellulose and nitrogenous pesticides, including simazine (Figure 11-lower right inset). The sediment here is also associated with bacterial assemblages that rapidly metabolize the nitrogenous pesticide (McConnell et al 2004).

It is possible that the Chesapeake Bay transect can be the result of the three processes of TNT transformation originally described in the literature. In the freshwater end-member, TNT is

metabolized dissimilatorily and not as a carbon or nitrogen source. The high incorporation efficiencies may be an artifact of binding of aminotoluenes to particulate matter (or even the bacteria themselves). The low TNT mineralization rates measured are consistent with this hypothesis. Some areas of exceptionally high mineralization rates may be the result of rapid lignin metabolism. These areas include the Pawtuxant River mouth and the zones of convergence between water masses in Kahana Bay. Bacterial assemblages in much of the estuarine and marine ecosystems may be assimilating TNT, similarly to that of other common organic nitrogen source, like amino acids. This would explain the covariance between TNT mineralization and heterotrophic production (which is measured by amino acid incorporation). If TNT is preferentially incorporated into bacterial proteins rather than catabolized for energy, as are amino acids, then the mineralization rates measured may actually be remineralization of bacterial macromolecules by protozoan grazers. Rates of remineralization would likely correlate with bacterial production rates needed to support growth of the protozoan grazer population.

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